

Introduction of biological and active Modified Atmosphere Packaging through microorganisms

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Introduction of biological and active Modified Atmosphere
Packaging through microorganisms

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Abstract

Modified Atmosphere Packaging (MAP) is a technique for modifying internal gas atmosphere of the food package to slow deteriorative reactions inside the package and prolong shelf life of the product. However, due to current limitations of this passive technology, there might be some opportunities for introduction of active MAP through microorganisms. Therefore, the study is conducted on investigation of different types of packaging structures, microorganisms and packaging processes to find the optimum combination of these three factors for creating an active modified atmosphere inside a package. Among non-pathogenic microorganisms, three different groups, which are able to consume oxygen and produce carbon dioxide, are evaluated. These microorganisms, including 1) microaerophiles, 2) facultative fermentative and 3) probiotics, might be able to create modified atmosphere inside the package. They can be incorporated into the packaging structure, either into a closure or into the packaging layer. Moreover, it is also concluded that the physiological state of the microorganisms, either in the form of vegetative cells or spores, has to be in correlation with the packaging process. During the theoretical evaluation of this project, *Saccharomyces boulardii* is selected in its vegetative form as the potential microorganism to be incorporated into the closure of an orange juice package. Additionally, in the case of applying microorganisms into the packaging film for the creation of MAP, it is investigated whether *Bacillus amyloliquefaciens* can be the potential microorganism. However, further investigation through laboratory experiments is needed to be able to determine the best conditions for creating MAP by these microorganisms. Different aspects of introducing this method to the market, including safety regulations, environmental aspects and consumer benefits, are furthermore evaluated.

List of abbreviations used in the report:

MAP: Modified Atmosphere Packaging

CAP: Controlled Atmosphere Packaging

MO: Microorganism

OS: Oxygen Scavenger

WRAP: Waste and Resources Action Programme

EFSA: European Food Safety Authority

FDA: Food and Drug Administration

QPS: Qualified Presumption of Safety

GRAS: Generally Recognized as Safe

SML: specific Migration Limit

OML: Overall Migration Limit

PET: Poly Ethylene Terephthalate

PETG: Poly Ethylene Terephthalate, 1, 4-cyclohexane dim ethanol

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1. Introduction

1.1 Why do we need Modified Atmosphere Packaging

Food production has a great burden on the environment and an important contributing factor is food spoilage due to passed expiry date. Although there is no reliable source of data related to generation of food waste, according to Waste and Resources Action Programme (WRAP), 30 percent of the food products purchased by consumers end up in the trash bin in the United Kingdom. (WRAP, 2008) Passed expiry date, aesthetic appearance and moldiness are of main identified reasons for food wastage (Bakas & Herczeg, 2010). Spoilage not only leads to wasting of the food product, but also results in wasting of the whole energy during the supply chain of the product, including the package (Guidelines, 2010). Packaging has a critical role for keeping the shelf life of the food product by protecting it from different factors including light, oxygen, vapor, microbial and chemical contamination either actively or passively (William, et al., 2008). Therefore, by increasing the shelf life of the product through packaging, environmental impact will be reduced.

On the other hand, development of the food-packaging systems in the recent years has been in response to trends in customer preferences toward mildly preserved food products with fresh-like quality and prolonged shelf life. In addition, new trends in marketing such as globalization of the market and centralization of activities result in increase of distribution distances. These trends are putting demands on the packaging industry to increase the shelf life of the products while maintaining their quality and safety. (Vermeiren, et al., 1999); (Kruijf, et al., 2010) Acceptable quality of the product from an organoleptic and safety point of view depends on four important factors including *processing, formulation, packaging and storage conditions*. Relative importance of these factors depends on the perishability of the food product. As an example regarding importance of packaging factor, shelf life of a perishable food which is generally under 14 days can be increased up to 90 days by use of modified atmosphere packaging (MAP). (Galic, et al., 2011)

MAP, which is a method of prolonging shelf life, can be created either by active or passive modification process (Yam & Lee, 1995). This technique can maintain the product's quality and extend its shelf life by slowing chemical and biochemical deteriorative reactions. MAP can also slow down or prevent the growth of spoilage organisms and contribute to preservation, food safety and increasing the shelf life of the product. In this technique the modification process tries to decrease the amount of oxygen in order to mainly slow down the growth of aerobic organisms and also to reduce the oxidation rate. Oxygen can be replaced by different gases such as nitrogen and carbon dioxide, which can delay the growth of facultative anaerobes (García-Esteban, et al., 2004). Atmosphere modification within the package is influenced by atmospheric composition and initial free volume inside the package, commodity respiration rate and film diffusion characteristics (Kader, et al., 1989)

1.2 Current problems of MAP technology

Although modified atmosphere packages or vacuum packages are used to appropriately pack oxygen sensitive food products, these methods do not completely remove the residual oxygen in the head space of the package or the dissolved oxygen in the product. Moreover, these packaging techniques are not capable of active scavenging of permeated oxygen in the head space of the package. In contrast, active oxygen scavengers can overcome these limitations. (Vermeiren, et al., 1999); (De Kruijf, et al., 2002)

Leakage is characterized by gases that diffuse through the package. It is usually caused by permeable packaging materials, mechanical damage during transport and handling or by inappropriate sealing. As a result, in most cases oxygen enters the package and carbon dioxide escapes. This changes the gas atmosphere inside the package. Therefore, the packed product may lose quality and its shelf life could be decreased. Leakage of the MAP is usually difficult to detect and it is determined only after food has been spoiled. (Randell, et al., 1995) It also needs to be mentioned that indicators can be used in MAP to check gas mixture and detect machine faults; however, according to Ahvenainen, et al (1995), there are some deficiencies in these indicators. Also, despite a leakage in the package, oxygen and carbon dioxide might be maintained at the same level in the head space of the package. It can be due to oxygen consumption and CO₂ production by the spoilage microorganism in the product. (Kruijf, et al., 2010) Moreover, although indicators can detect non-integrity of the package at the time of packaging process, even intact packages leak. (Randell, et al., 1995); (Kruijf, et al., 2010) It might be due to absorbance of the volatile compounds of the head space into the sealing area and therefore gradual weakening of the seals (Vermeiren, et al., 1999). In addition, due to higher permeability of CO₂ than O₂, it needs to be actively produced to maintain the desired interior atmosphere of the package (De Kruijf, et al., 2002). Therefore, to solve the current problems of MAP, active MAP might be a good solution.

2. Purpose

The purpose of this thesis is to review what previous studies have found about how it is possible to create an active modified atmosphere package (MAP) through introducing non-pathogenic microorganisms into a package. Also it is aimed to make pre-studies on theoretical basis to investigate the possibilities and the advantages of implementing non-pathogenic microorganisms particularly probiotics for providing an oxygen poor/carbon dioxide rich atmosphere inside a package. These probiotics are aimed to be incorporated into the packaging layer or placed into the closure of a package during the practical experiments in the future. In addition, evaluation of possible interactions of probiotics with the product and assessment of resulting effects on safety, nutritional value and sensory properties of the product will be important to investigate in the future studies.

3. Limitation / Focus

In this project different types of microorganisms will be theoretically evaluated to find the potential microorganisms which are capable of creating modified atmosphere inside the package. These potential microorganisms will be incorporated into the closure to be released into the product in the future studies. Concerning the packaging material, focus will mainly be on closures covered by a gas permeable layer constructed from fibers, papers, waxes and polymers such as paraffin. Closures containing microorganisms might be applicable for packages of several types of food products, but in this study package of beverages such as fruit juice will be considered. In addition, since only primary package is considered, financial and logistical aspects, which are relying on a holistic view towards the whole supply chain, including secondary and tertiary packages, will not be evaluated in this project.

4. Method

In order to investigate the feasibility of the idea pre-studies on theoretical basis were made to get deep knowledge in previous studies on active MAP (what is done for creating active MAP before). In this regard, different keywords such as active modified atmosphere packaging and application of microorganisms in the packaging industry were used in databases such as ScienceDirect, Elsevier and PubMed. Then based on the researches, the study further sets out on 1) investigation of microorganisms which are capable of creating MAP and 2) packaging materials which allow microorganisms activity inside the package. Different types of packaging structures, microorganisms and packaging processes were evaluated to find the optimum combination of these three factors for creating a modified atmosphere inside the package. Based on these investigations through the literature review and personal communications, two types of microorganisms into two packaging structures were suggested for the proposed idea.

5. Theoretical background

5.1 Active Packaging; Oxygen Scavengers

Passive package only acts as a protective barrier, whereas active package controls and reacts to the external variations and constantly changing interior atmosphere of the package to maintain the quality of the product and prolong its shelf life (Kruijf, et al., 2010). Active packaging is distinguished into several systems, including active releasing systems such as flavor emitters and active absorbing systems like oxygen scavengers, which will be discussed in greater detail. (William, et al., 2008)

Much food spoils rapidly due to oxidation and growth of aerobic microorganisms. Microbial growth results in changes in texture, color, flavor and nutritional value of the food. These changes

can reduce the shelf life and safety of the product. (García-Esteban, et al., 2004) In order to solve this problem, oxygen scavengers are used as an adjunct for oxygen barrier packaging films and contribute to controlling of the interior atmosphere of the package (Miller, et al., 2003). There are several active oxygen scavengers such as iron powder, ascorbic acid and enzyme in the form of a sachet (Singh, et al., 2011). As an alternative to the sachets, oxygen scavengers can be suspended into the closures and crown caps or blended with the packaging materials (Dawson, 2003). These materials can be used as a layer in a multilayer package (e.g. Zero₂ and Amosorb) or as a liner in a closure (i.e. Darex, Smartcap and PureSeal). (Kaufman, et al., 2000); (Rooney, 2005); (Vermeiren, et al., 1999); (Suppakul, et al., 2003); (Miller, et al., 2003). Oxygen scavenging adhesive labels such as FreshMax, FreshCard and ATCO1 labels are also of further developments which are applied into the lidding film or inner wall of the package (Vermeiren, et al., 1999)

5.2 Consumer Benefits and Convenience Aspects of Active Packaging

Competition for shelf space is increasing and moving from media to the point of purchase which makes packaging more important than before in order to meet consumers' expectations. On the other hand, increasing time pressured life style is creating a demand for new and innovative packaging for food and beverage products. A common pitfall when a new technique of packaging is introduced is to consider it as a successful technology with no consideration of important parameters to the consumer at each stage of product's lifecycle. For successful introduction of a new product, the consumer benefits need to be the leading factor. A major area in which active packaging can be developed is helping customers to achieve things in better, faster or different ways by improving the level of convenience for them. By adding a new active packaging concept in the most important stages of packaging, which are purchase, use and disposal, a distinct utility proposition from existing products can be created. In this regard, different convenience functionalities such as self-cooling and self-heating packaging have been introduced for beverage industry by active packaging. (Kerry & Butler, 2008)

5.3 European Market for Active Packaging

Virtually any kinds of products, even organically grown foods, need packaging as an essential market component for protection, handling and storage. As a result the food and beverage industry are continuously introducing new technologies which improve the quality of the products and enhances their profitability by prolonging their shelf lives. The global market of new packaging technologies is divided into different technology applications of controlled, active and intelligent packaging. Among all, controlled/modified atmosphere packaging has the largest segment of the market comprising 45.4% in 2008 and is estimated to have a slightly decreased percent to 40.5% in 2013.

Over the past decade, the current status of the traditional packaging has been challenged due to significant growth of active and intelligent packaging. These new technologies were first

introduced to the Japanese market in the mid-1970s and then raised the attention of markets in Europe and the US in the mid-1990s. Approximately 27% of the global market in 2008 was comprised by active packaging but this segment is estimated to be slightly decreased to 26.9% by 2013. The value for this technology has been \$4.6 billion in 2008 and is estimated to reach \$6.4 billion by 2013. [Figure 1] Oxygen scavengers as the leading technology of active packaging has been accounting for 37% of global market for this technology in 2005. (Restuccia, et al., 2010)

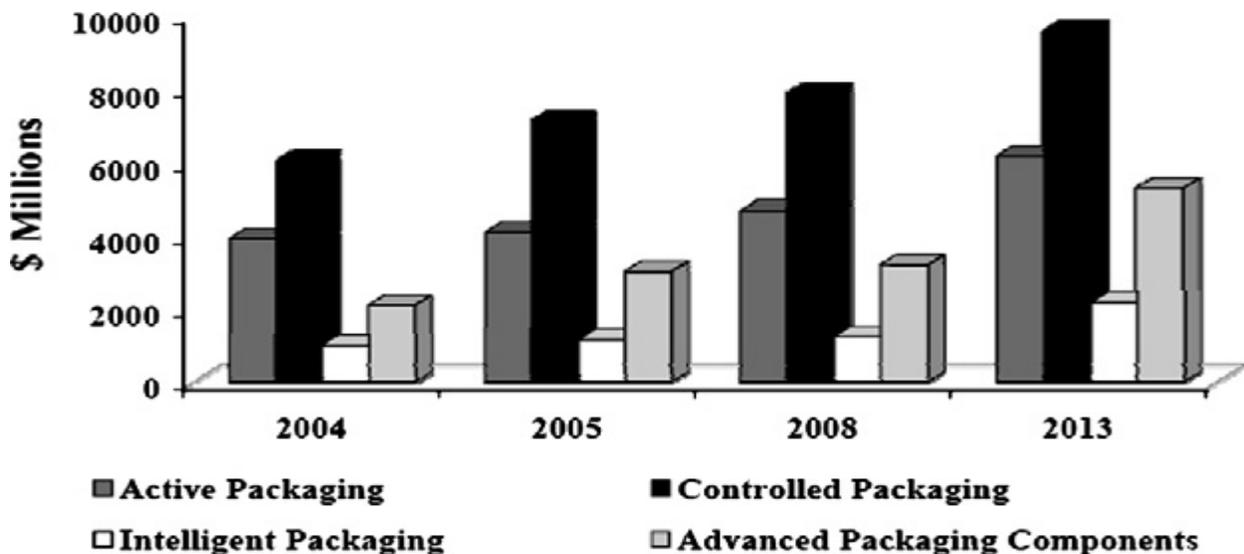


Figure 1: Growth of active, controlled, and intelligent packaging for the food and beverage industry 2004–2013 (\$ millions). (Restuccia, et al., 2010)

Active packaging is developing due to the growing demand for ready-prepared and packaged foods. It is being driven by consumer desires for freshness, convenience, food safety, quality as well as increased shelf life in the food and beverage market. (Restuccia, et al., 2010) However, differences in legislation all over the world have a major impact on market diffusion of active packaging. Considering penetration of active packaging in the EU market, EU is the only region across the world that has specific legislation for this technology. In addition, applications of active releasing materials were permitted only after formulation of EU framework regulation 1935/2004. Late formulation of the legislation for active packaging has delayed the introduction of this technology into the European food market. (William, et al., 2008)

In addition, accidental ingestion of active components of the sachets such as oxygen scavengers has hampered their commercial success in Europe and North America. However, oxygen scavenging adhesive labels and incorporation of oxygen scavengers into the laminated films have been developed to enhance commercial acceptance of this technology. Similarly, incorporation of oxygen scavengers into the bottle closures, crowns and caps are examples of removing accidental ingestion of oxygen scavengers used in beverage industry which is a huge market for application

of this technology. The PureSeal oxygen scavenging bottle crowns, cobalt catalyzed oxygen scavenger films and light activated ZerO₂ oxygen scavenger materials are a few examples of active packaging aimed at beverage market. (Kerry & Butler, 2008)

The next hurdle for active packaging in Europe is relatively low acceptance and conservative attribute of European consumers towards active packaging (William, et al., 2008). In contrast to Japan, where oxygen scavengers have been a commercial success due to acceptance of innovative packaging, European consumers have no affinity to this type of technology and also they are not willing to pay extra costs for it (Kerry & Butler, 2008); (William, et al., 2008). Considering costs, with broader application and scaling-up the production, the costs of this technology will be significantly reduced. However, consumer, food producer and retailer acceptance is needed to enable an application on a broad scale. (Restuccia, et al., 2010)

Therefore, the development efforts in Europe will be expected to be for cost reduction, acquiring approvals for active packaging concepts and identifying the benefits that will justify the additional costs of the package (William, et al., 2008). With respect to this, the ACTIPAK project, which is a European commission funded project, demonstrated that consumers in the European countries are open to development of active packaging when it provides safe material and unambiguous information for them (Restuccia, et al., 2010); (Kerry & Butler, 2008). For consumer acceptance the nature of applied technology and releasing compounds is very crucial and it is not gained only by extension of product's shelf life (William, et al., 2008).

To conclude, consumer demands (for fresher, safer, more convenient and minimally processed products) along with industry's demand (for safety pack foods while maintaining the quality) present a bright future for active packaging. Therefore, growing interest for application of this kind of technology is expected in the coming years. (William, et al., 2008) (Restuccia, et al., 2010)

5.4 Safety regulations of Active Packaging

In contrary to the US, Japan and Australia, where active packaging is already a commercial success, EU has applied only few of these systems and the legislation is stricter than in other parts of the world (William, et al., 2008). The regulatory concepts about food contact materials in Europe not only in details but also in fundamental approaches differs from in the US. In the European approach, all the materials should be accurately cleared based on a toxicological evaluation of the listed substances and should be publicized in regulations, whereas in the US substances that are not likely to raise any public health concern based on the analytical chemistry data, or those which may not be expected to become food components are cleared. In fact, while the US approach gives reliability to the idea that the dosage makes the poison and toxicological justification is minimized by minimal exposure; the principle of the European approach is that regardless of the level of exposure there must be a toxicological data on all substances (Restuccia, et al., 2010).

In the US, there are no specific regulatory concepts that differ from the requirements put on conventional packaging materials as long as the packaging material is not intended to have a technical effect on, or add any substance to, the food. In contrast, if active packaging materials have a technical effect on the food product, or they are directly added to it, the materials would be subjected to the stricter FDA (Food and Drug Administration) regulations. In other words, released substances are allowed to change the composition of the food product if they are authorized by FDA as food additive. In contrary to the US, the regulation of such technologies in Europe is under development. Primarily, European food contact legislation was applied in individual member states. However, to overcome barriers to trade and create a single market legislation was harmonized by the EU. This resulted in formation of a new regulation for food contact materials (1935/2004/EC). This regulation, which also applies to active packaging system, protects consumers' health by ensuring that no food-contact materials can facilitate chemical reactions that may change the organoleptic properties or composition of the food product. The main aspects of this framework regulation first require all new active packaging systems primarily to be evaluated by regulations of ESFA (European Food Safety Authority). Then based on the results of this evaluation, a petitioner authorization for the submitted active ingredients, which will be entered the regulation, will be granted by the commission (DG SANCO) (Restuccia, et al., 2010). ESFA guidelines contain the following issues:

- I. Three risks related to the dietary exposure of chemicals including:
 - Migration of active components; Since it may not be easy to determine and demonstrate that migration of the active substances do not endanger human health, dedicated migration methods are required;
 - Degradation of active components and migration of their reaction products;
 - Toxicological properties of the active substances;
- II. Efficacy of the active packaging; Active packaging should have the intended effect on the food product. If there is no effect, there is no benefit and it results in misleading of the consumer. So it should not be used even if there is no health concern. Antimicrobial components intended to be released into the food product need evidence of their efficacy and must be approved as food additive. Also the status of the food additive for the releaser is crucial.
- III. Proper labeling of the released substances, their amount and their functionality; Labeling should comply with the food additive directive and must meet requirements of regulation 2004/1935, directive 89/109/EEC and directive 79/112/EEC. As an example, to allow identification of active materials and non-edible parts by consumer, specific requirements according to Regulation 450/2009/EC must be considered. These parts must be labeled with the words of 'DO NOT EAT' or by symbols. (William, et al., 2008); (Restuccia, et al., 2010)

Moreover, all passive parts of the active packaging systems are initially subjected to preexisting FCM (Food Contact Material) legislation (William, et al., 2008); (Restuccia, et al., 2010) . As an example, since many active packaging systems are applied in packaging materials composed of plastic, monomer regulation must be primarily met (Restuccia, et al., 2010).

5.5 Microorganisms as oxygen scavenger

In addition to chemical oxygen scavengers introduced in previous sections, use of microorganisms as oxygen scavengers has been investigated in several studies. A study conducted by Altieri. C et al (2004) has proposed a method to produce an oxygen scavenger film using microorganisms as active ingredients. In this study hydroxyl ethyl cellulose (HEC) and polyvinyl alcohol (PVOH) were used to entrap aerobic microorganisms such as *Kocuria varians* and *Pichia subpelliculosa* and the highest respiratory efficiency was evaluated.

Another study by Anthierens. T et al (2011) has described a model system for using endospore-forming bacteria genus *Bacillus amyloliquefaciens* as the active ingredient for oxygen scavenging of oxygen sensitive foods. Since microorganisms are needed to be heat stable during the heat process of integrity with the polymer layer, two strategies were applied in this study to meet this requirement. Firstly, they tried to keep the bacteria alive against the heat and anaerobic condition by spore formation. Endospores have higher stability to heat, chemical substances and nutritional diversity than vegetative cells. Secondly, PET (Poly Ethylene Terephthalate) was replaced by PETG (poly ethylene terephthalate, 1, 4-cyclohexane dim ethanol). PETG has lower melting point due to its less crystalline and more amorphous structure. As a result, less temperature was needed to be applied on the microorganism during the heating step of manufacturing process. Higher moisture uptake is an additional advantage of PETG materials in comparison with PET films. Therefore, moisture as a growth requirement for the microorganism was provided. Two processes occur during activation of spores as an oxygen scavenger. First, uptake of moisture by PETG that takes 1 to 2 days. Second, activation of spores. Oxygen scavenging rate is in relation to oxygen concentration and respiratory metabolism of the spores. (Anthierens, et al., 2011)

In the study by Anthierens. T et al (2011) excluding transmission of oxygen and moisture, there was no contact between bacterial layer and the product. Hence, microbial up take of nutrition from the product and release of metabolites to the product was limited. As a result, metabolic activity and oxygen absorption rate (OAR) of the bacteria was gradually decreasing for two reasons. Firstly, depletion of the nutritional sources of carbon and nitrogen which came from residues of spore medium in the spore preparation step. Secondly, self-toxicity of the bacteria due to accumulation of CO₂, ethanol or other metabolites. (Altieria, et al., 2004); (Anthierens, et al., 2011)

A patent invented by Hopkins & Banasiak (1990) has utilized alcohol oxidase enzyme derived from methylotrophic yeast to scavenge oxygen. This scavenging system comprises a gaseous alcohol such as ethanol introduced together with dried whole cells, broken cells or cell extracts of

Pichia. pastoris which contain glucose oxidase enzyme. The alcohol oxidase derived from the yeast catalyzes the reaction between oxygen and gaseous alcohol resulting in removing oxygen from the package. This system can be encapsulated with a gas permeable layer or can be directly placed into the container.

Moreover, another study of applying microorganisms to the packaging material for their oxygen scavenging activity has been conducted by Gist- Brocades (1994). In order to minimize oxidative reactions in pasteurized and non-pasteurized containers of water-containing products such as beverages, immobilized yeasts on a food-grade solid material were introduced. In this study slurry mixture of *Saccharomyces cerevisiae* and molten immobilizing material were fixed on the inside of stopper or cork of the container. (Gist-Brocades, 1994)

However, Active MAP comprises an active oxygen scavenger along with an active carbon dioxide emitter. Therefore, although active oxygen scavengers such as microorganisms assist shelf life extension of the product, they cannot be used as substitute of Active MAP.

6. Analysis and Discussion

6.1 Potential microorganisms

As a part of this project, certain types of microorganisms that are capable of creating active modified atmosphere inside the package were evaluated. These microorganisms can be mainly activated as oxygen starts to penetrate the package. Then, they act as oxygen scavengers, preventing oxygen to be available for other aerobic microorganisms. These microorganisms also prevent increasing redox potential and chemical reactions which lead to spoilage. (Altieria, et al., 2004); (Anthierens, et al., 2011); (Adams & Moss, 2008) In addition, they can release carbon dioxide as a reaction end product to the interior atmosphere of the package.

Food requirements that put demands on the package selection, including oxygen scavengers, are divided into 1) head space scavenging and 2) barrier enhancement. Typically both requirements are considered, but the economical approach is to use oxygen scavengers to resolve one problem or the other. (Rooney, 2005) Therefore, application of microorganisms can be developed in two lines, whether they are added into the package as an insert for head space scavenging (e.g. sachets) or designed to be part of the packaging structure for barrier enhancement which will be discussed in the following paragraphs.

6.1.1 Microorganisms incorporated into a sachet

In order to keep microorganisms alive before and after oxygen penetration, they need to be compatible with both aerobic and anaerobic conditions. In the following paragraph three groups of microorganisms which meet this requirement will be introduced. Depending on the results of

the evaluation, one of three strategies can be taken in order to create MAP by incorporation of microorganisms into the package. Figure 5

1. Facultative fermentative yeasts

If there was no limitation for release of fermentation end-products to the product regarding sensory alterations, safety and nutritional value considerations, applying facultative fermentative yeasts results in several advantages as below. To assure these, released metabolites must be approved as food additive by the FDA or must be verified by EFSA.

- a. **Releasing fermentation end products including CO₂, weak acids, ethanol and bacteriocins into the product:** These metabolites contribute postponing spoilage and creating longer shelf life beyond oxygen scavenger activity of the microorganism. Health aspects and nutritional improvements such as increasing digestibility of the nutrition and vitamin C saving are other advantages of these metabolites. Fermentation also enhances food availability and lessens malnutrition in many developing countries. However, quantities of these beneficial end products by fermentative yeasts inside the package need to be investigated. Thus, despite potential health benefits of these metabolites, significance of their benefit on the food product with the proposed packages cannot be discussed.
- b. **Preventing self-toxicity of the oxygen scavenger bacteria by allowing accumulated reaction end-products to be released into the product.**

2. Microaerophiles

If it was concluded that the quality of the product can be negatively affected by fermentation end products, the contact of the product with bacterial film would be limited to prevent release of reaction end-product to the product. In this case usage of fermentative bacteria with fermentation process, which is a demanding process, does not result in any additional benefit. Therefore, it is preferred to focus on non-fermentative bacteria such as microaerophiles. These microorganisms can survive under anaerobic conditions but grow best in low oxygen concentration. They are specialized for growth in oxygen-limited environment. They have oxidative metabolic gearing, which makes them adaptable with both aerobic and anaerobic conditions. Scavenged oxygen activates them and stimulates minor metabolic processes resulting in producing minimum metabolites as we wish. (Altieria, et al., 2004)

3. Facultative anaerobic probiotic microorganisms

Due to increasing popularity of the probiotics, supplementation of food products with probiotic bacteria is a rapidly growing segment. In order to provide beneficial health effects on the intestinal tract of the host, these microorganisms, which are non-pathogenic,

live and healthy bacteria, can be added to the food products. Microorganisms must meet several requirements to be considered as probiotic. These requirements include ability of the microorganism to inhibit growth of harmful organisms, survive in sufficient number and tolerate acidic environment of the stomach. (Thirabunyanon & Thongwittaya, 2011) Probiotics improve intestinal microbial balance and produce antimicrobial substances toward pathogens (Heller, 2011); (Logan, 2011). Bifidobacteria and Lactic acid bacteria (LAB) are the primary types of microorganisms used as probiotics in the commercial products but certain types of yeasts and bacilli may also be used. There are different delivery systems for probiotics including drinks, capsules and powders. (Parkes, 2007) Available forms of probiotics include: 1) heat-dried culture supernatants, 2) capsules of freeze-dried cultures and 3) those that are mixed in diary food (such as yogurts) or other food (chocolate, wafers). (McFarland, 2010) Although capsules and powders contain high number of probiotics, they may lose them during the storage, while the problem with products containing probiotics is survival of bacteria during the manufacturing process. Preparation can result in significant inconsistency in bacterial number, fluctuating from 150 million to 450 billion per daily dose (Parkes, 2007). In the following paragraph advantages of applying probiotic bacteria into the package, compared to their direct addition to the product, which is currently used in the commercial products, is discussed:

- a. **Providing possibility of probiotic health claim for a wider variety of food products:** Stability of the probiotics in a food product during manufacturing and storage is a key requirement. All essential properties of probiotics for promoting health effects of the product must be retained during the storage and manufacturing steps. There are several factors determining the ability of the probiotics to survive in the product and to start activity as entering the host's gastrointestinal track. These factors include possible interactions of probiotic organisms with the food matrix, physiologic state of the probiotic strains, physical form and chemical composition of the food (e.g. acidity, oxygen content). (Heller, 2011)

Therefore, though possible incorporation of probiotics to any type of food, they are mainly added to the fermented dairy products (da Cruz, et al., 2007). The manufacturing process of these products has been already optimized to guarantee viability of fermentation microorganisms. Thus, to assure viability of probiotics, only minor changes need to be introduced into existing manufacturing processes of these products (Heller, 2011). By applying probiotic organisms as a part of the package, production of products with probiotic health claim would not be only limited to the fermented dairy products. As a result, producing wider variety of non-fermented food product with probiotic health claim would be possible.

- b. **Minimizing negative interactions of probiotics and starter microorganisms in the probiotic containing fermented products:** Dairy products form the largest segment of the market of probiotic containing products and are well adapted to promote health image of the probiotics. In production of probiotic fermented dairy products, both synergistic and antagonistic interactions between starter microorganisms and probiotics are very important. In an ideal probiotic dairy product, microorganisms are isolated from the typical flora and antagonistic interactions are minimized. (Heller, 2011) Therefore, it can be concluded that in order to improve survival of probiotic cells in sufficient amount, their adverse interactions with the starter organisms or food matrix can be minimized through isolation of probiotic strains into the package.
- c. **Facilitating the manufacturing process:** It provides convenience for the manufacturer and reduces related costs of process modification. In order to fulfill all requirements for survival of probiotics, the manufacturing processes of supplemented foods with probiotics need to be modified. (Heller, 2011); (Kerry & Butler, 2008) Therefore, by the new type of the package, containing probiotics, less process modification is needed for manufacturing of probiotic containing products.

Moreover, alternative packaging material for better protection or improving shelf life of the product is not the only value which packaging can add for cost reduction. In other words, the effect of packaging on the production process of the probiotic containing products might be a good solution for cost reduction. As an example, designing an optimized process for keeping the probiotic bacteria alive until the point of consumption not only costs, but may also affect the critical steps of the process such as heating for reducing the spoilage bacteria. Therefore, optimization may indirectly affect the potential shelf life of the product. While in the case of adding probiotics at the end of production process as a package layer or a sachet, critical steps of the process would not be limited and costs for process optimization might be reduced. In addition, effective dose and viability of probiotics will be ensured as they are remained separated from the product until the point of consumption (Kerry & Butler, 2008).

- d. **Convenience for people who used to take tablets of probiotic:** This is achievable by providing symbiotic containing drinks for them by replacing tablets of probiotics by symbiotic containing drinks. (Kerry & Butler, 2008)
- e. **No compromising on taste and ingredients of the product:** Since probiotics are aimed to be added to the product just prior to consumption, interaction of probiotics with the food matrix is minimized. (Kerry & Butler, 2008)

Therefore, particular probiotics can actively absorb oxygen and release carbon dioxide into the interior atmosphere of the package. Beside oxygen scavenging activity of probiotics, release of these microorganisms to the product at the time of consumption is an additional benefit.

6.1.1.1 Packaging material for incorporation of microorganisms into the sachet

Head space scavenging is advantageous typically for beverages and porous products where residual oxygen is not economically reduced to the necessary level by evacuation or gas flashing. It is beneficial in beverages with froth containing air bubbles that are not readily deoxygenated. Although sachets are satisfactory for head space scavenging of a wide range of food products, they are unsuitable to be used in food products with high water activity such as beverages. These active ingredients will lose their capability when they become wet. (Rooney, 2005); (Kerry & Butler, 2008) Thus active ingredients incorporated into the closures and polymers have wider use for these types of high humidity products respectably for both purpose of barrier enhancement and head space scavenging (Dawson, 2003).

In order to modify headspace atmosphere of the beverages, active components need to react with the atmospheric gases without any reaction with the beverage. To achieve this, microorganisms are incorporated into the closure in two forms. In the first method the sachet containing microorganisms is attached to the inside of the closure and is covered with a membrane. The membrane, which is permeable toward gases and vapor, separates the active ingredients from the beverage. The second method involves incorporation of the microorganisms into the coating layer inside the closure. This layer needs to be permeable itself (e.g. SmartCap and PureSeal). (Dawson, 2003) The first method is desirable for this project.

6.1.2 Microorganisms integrated into the packaging material

Microorganisms can be integrated into the package's wall, either mixed with the packaging material forming a uniform layer or attached as a separate layer of a multilayer package. Heat liability of the incorporated biological compounds, such as microorganisms, during the high pressure and temperature production process of plastic containers is a significant limitation for their activity as OS (oxygen scavenger). Therefore, currently available biological systems of OS are incorporated in a low melting paraffin, gels or water soluble polymers. For this reason application of these biological systems into the food packaging is limited. As a result, in order to make microorganisms suitable to be directly incorporated in a plastic material, they need to be heat-stable during the procedure of pressing and incorporation to the packaging material. (Anthierens, et al., 2011) To achieve this, only sporulated microorganisms are able to survive at high temperature of 210 °C. Hence, to apply microorganisms into the packaging layers, focus will be on spore-forming or high temperature resistant microorganisms. Therefore, three types of previously mentioned microorganisms suggested to be incorporated into the sachet (section 6.1.1), need to be also spore-former to be applied into the packaging material:

1- Spore-forming aerobic bacteria or heat resistant microaerophiles [Figure 2].

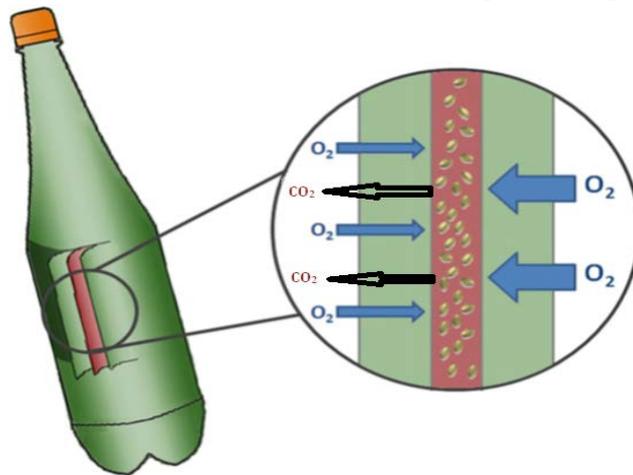


Figure 2: Creating MAP via heat resistant microaerophiles or spore-forming aerobic bacteria. These bacteria might be able to scavenge the oxygen in the head space of the package and emit CO₂ to the interior atmosphere of the package. Figure modified from (Anthierens, et al., 2011).

2- Spore-forming aerobic fermentative microorganisms [Figure 3].

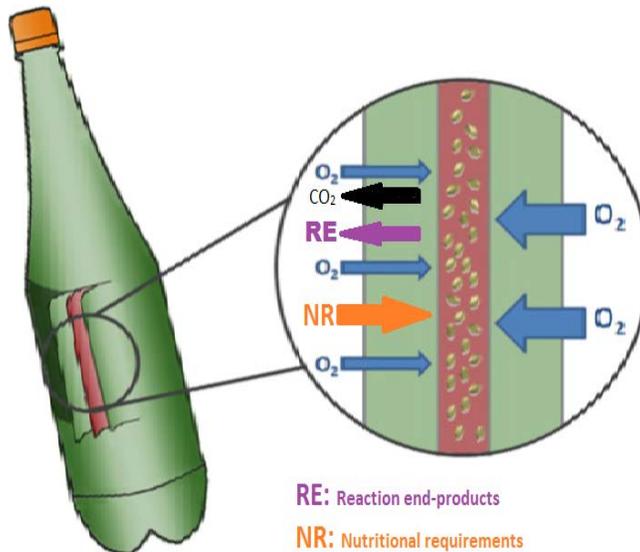


Figure 3: Creating MAP via spore-forming aerobic fermentative microorganisms. These bacteria might be able to absorb their nutritional requirements from the product, scavenge the oxygen in the head space of the package and release CO₂ and other fermentation end products to the interior atmosphere of the package. Figure modified from (Anthierens, et al., 2011).

3- Spore-forming aerobic probiotic microorganisms: Even though spore-forming bacteria are not identified as GRAS (Generally Recognized as Safe) by the FDA to be used as probiotics, *Bacillus subtilis* species have been identified as QPS by EFSA. They have been approved as probiotic food supplement in Italy. (Beek & Brul, 2010); (Thirabunyanon & Thongwittaya, 2011). *Bacillus*

species as a probiotic are widely marketed in Asia and some products are purposely supplemented with these strains (Logan, 2011); (Beek & Brul, 2010). By placing a probiotic layer into the package, modified atmosphere inside the package might be created to additionally improve the quality of the product. The bacterial layer of probiotics can be placed in to the package in two forms: [Figure 4]

- A) Probiotic spores placed as a middle layer of the package: Once spores are activated to the vegetative cells, they partly do fermentation for creating MAP and partly migrates through the permeable thin layer of PET to be added to the product. This method might work as already existing techniques for releasing antimicrobials that are attached to the film surface and are continuously freed to the product. However, it would not be economically beneficial that probiotics are released into the product, as the scavenging effect only lasts for several minutes to hours.

- B) Probiotic spores as the interior layer of the package: As spores are activated, they start activity to create modified atmosphere inside the package. Then at the time of consumption of the product the bacterial layer, which is covered by an edible film (e.g. starch-based materials preferably prebiotics such as inulin), can be separated by the consumer and mixed with the product. The attached edible layer might be possible to be provided by the Royal Veterinary and Agricultural University in Denmark. This research institute is one of the leading research units for bio-based food packaging materials and is developing starch-based material for packaging beverages. (Dawson, 2003)

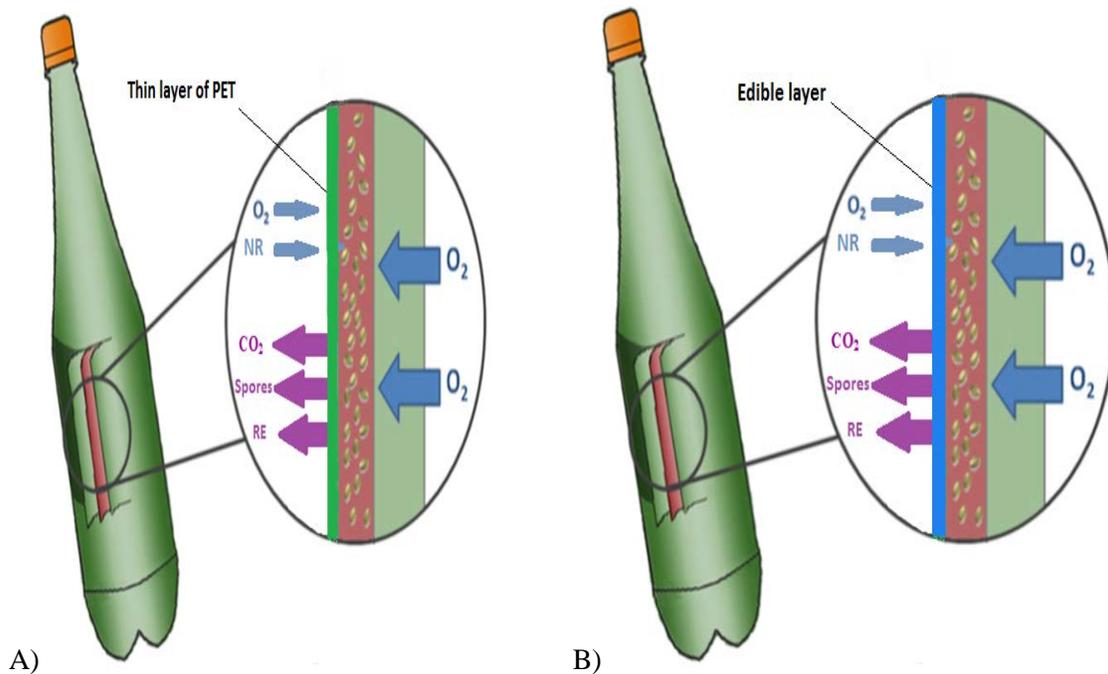


Figure 4: Probiotic bacteria for creating MAP inside the package are incorporated into the multilayer structure of the bottle. They absorb nutritional requirements from the product and release reaction end products to it. The probiotic bacteria as a layer of the package can be covered by A) Thin layer of PET to partly migrate to the product, B) Edible layer to be added to the product at the time of consumption by consumer. Figure modified from (Anthierens, et al., 2011).

B. amyloliquefaciens, which is a spore forming probiotic, might be the potential microorganism to be incorporated into the packaging film for creating MAP inside the package. (suggestion 1), (suggestion 2) or (suggestion 3). As spores of *B. amyloliquefaciens* are activated, this microorganism ferments the majority of carbon source to CO_2 , butanediol, acetate and lactate. *B. amyloliquefaciens* produces protease even in uncontrolled pH and releases natural antibiotics, α -Amylase, resulting in increased nutritional value of the product. It might also compete with spoilage bacteria by limiting available water and nutrition for their growth (Priest, et al., 1987); (Alam, et al., 1989).

It also needs to be considered that if there was any other possible procedure for incorporation of the microorganisms to the packaging film without high temperature; criteria for selection of optimal microorganisms might be changed. [Figure 5]

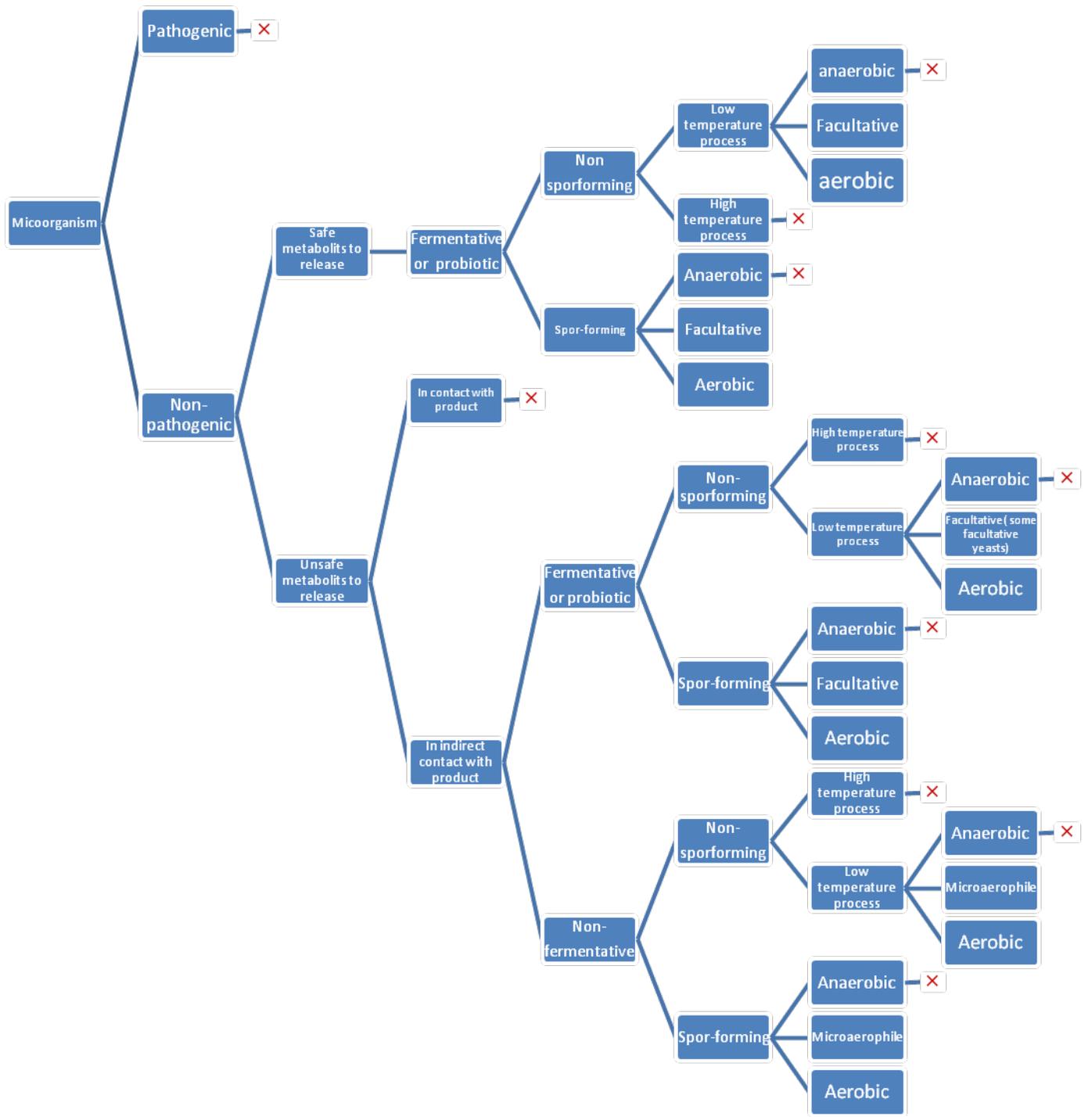


Figure 5: Procedure of selection of proper microorganism to be integrated into the package for creating MAP

6.1.2.1 Packaging material for incorporation of the microorganisms into the packaging layers

Barrier enhancement is commonly required for the food products with low headspace and thin barrier layer which need improving barrier properties within the packaging wall. An example for this is a PET beer bottle with low head space in which oxygen is permeated to the head space of the product through the PET layer. Barrier enhancement is also of assistance for products packed in an impermeable package, where oxygen needs to be prevented from entry through the closure. It can be achieved by applying active components into the bottle closure or within the bottle wall. Incorporation of microorganisms such as *B. amyloliquefaciens*, *K. varians* and *P. subpelliculosa* as active oxygen scavengers into the polymer layer of the package has been done so far. Among these studies, a study conducted by Anthierens. T et al (2011) suggested PETG as the potential packaging material for incorporation of microorganisms. So PETG might be a good option for future studies of this project. However, higher moisture uptake of the PETG than PET might be a challenging point for stability of the package particularly for high humidity foods such as orange juice. In addition, limited permeability of this material to only oxygen and moisture was resulting in self-toxification and metabolic activity limitations for the microorganisms. Therefore, a new and more permeable packaging material would allow the bacterial layer to be in more contact with the product. A permeable layer not only provides nutritional requirements for growth of the incorporated microorganisms, but may also prevent their self-toxification in the package layer by allowing metabolites to be released to the product. [Figure 3, 4]

To achieve this, according to Jannasch. P, (professor of polymer technology at Lund University), packaging material needs to be permeable toward:

1- Oxygen and carbon dioxide for suggestion number 1. [Figure 2]

2- Oxygen, carbon dioxide, nutrition and reaction end products for suggestion number 2. [Figure 3]

3- Oxygen, carbon dioxide, nutrition, reaction end products and partly to spores for suggestion number 3. [Figure 4]

A comparison of incorporation of microorganisms into a sachet (section 6.1.1) and into a packaging layer (6.1.2) will be also discussed in the following chapters (section 6.3.3).

6.2 Proposed method vs. current active packaging

In the following paragraphs, a comparison between already existing active ingredients and the proposed microorganisms as active ingredients will be provided.

While the main shortcoming of enzyme as an oxygen absorber is its sensitivity to the chemical-physical factors, there are two main problems with the sachet of iron powder. Firstly, migration of the active substances to the product, which must not endanger human health, might not be easy

to be determined and controlled (William, et al., 2008); (Altieria, et al., 2004). Secondly, in spite of the label of “Do not eat”, there is always a possibility of accidental ingestion of contents of the sachet (William, et al., 2008). In contrast, ingestion of particular microorganisms of probiotic in the proposed method of this project not only involves no risk, but also it is aimed to bring beneficial health properties to the product.

Furthermore, although oxygen dependent reactions and growth of spoilage aerobic bacteria are diminished by use of oxygen absorbents, there is a public health concern regarding growth of anaerobic pathogenic bacteria. Anaerobic environment inside the package created by the sachet of oxygen scavengers favors these microorganisms (Smith, et al., 1995). Also, the suppressing of the aerobic spoilage microorganisms will decrease the competition for growth of the pathogenic microorganisms (Galic, et al., 2011). On the other hand, carbon dioxide acts as an anti-spoilage factor through its effect on pH, growth of moulds and oxidative Gram-negative bacteria (Adams & Moss, 2008). The principal effect of increased carbon dioxide is an extension of the ‘lag’ phase of the growth of the bacterium and decreasing its logarithmic growth rate (Kerry & Butler, 2008) (Kader, et al., 1989); (Adams & Moss, 2008). It has bacteriostatic and fungistatic properties which are desirable for most of the food products which are commonly affected with molds. Therefore, a combination of oxygen absorbent and CO₂ rich atmosphere may inhibit the growth of all bacteria on these types of foods.

In addition, partial vacuum might be created due to oxygen absorbance. Hence, simultaneous release of carbon dioxide to the interior atmosphere of the package assists maintaining the pressure and prevents collapse of the flexible package. (Vermeiren, et al., 1999); (Kerry & Butler, 2008); (Rooney, 2005) Therefore, combination of both O₂ absorber/ CO₂ emitter is more effective than the sachet of oxygen absorber. However, it is worth to mention that growth of certain yeasts which are a major cause of spoilage in certain products can be stimulated by high levels of CO₂ (Kerry & Butler, 2008).

Dual-action oxygen scavenger/carbon dioxide emitter labels and sachets for different products including sponge cakes and snack food products have already been developed. These labels and sachets are developed by commercial manufacturers including Mitsubishi Gas Chemical Co. Ltd (Ageless™ type G), and Multisorb Technologies, Inc. (FreshPax r_ type M). They typically contain chemical substances such as ferrous carbonate and metal halide catalyst. In contrast, the new proposed cap of microorganisms, particularly the one with probiotics not only contains biological and natural components as active ingredients but also provides additional health benefits to the product. (Kerry & Butler, 2008)

Moreover, commercially available active ingredients such as Dual-action O₂ absorber/CO₂ emitters or oxygen scavengers listed in the [table 1](#) require activation prior to use or need to be covered in an airtight package to not be affected by oxygen before the intended use (Pereira de Abreu, et al., 2012). In contrast, particular microorganisms as active components neither need activation nor protection prior to use. These microorganisms can be activated from the beginning,

doing fermentation in the absence of oxygen and then start respiration as oxygen becomes available. Alternatively, they can be primarily inactivated in their stationary phase and then automatically become activated as oxygen and humidity is provided for their growth.

To sum up, due to several reasons it can be concluded that implementation of microorganisms for creating MAP might be an interesting alternative to the current MAP or sachet of oxygen absorbent/CO₂ emitters. These reasons include: limitations and low efficiency of oxygen scavenging packaging films, current limitations of dual-action sachets and beneficial health effects of the probiotics. In other words, it would be advantageous if nonpathogenic microorganisms preferably probiotics, which represent natural ingredients, protect the product. (Singh, et al., 2011); (Vermeiren, et al., 1999); (Gist-Brocades, 1994) This technique can be combined with MAP or used alone. The latter case could be considered as a substitute for MAP and may allow higher production speed of packaging due to elimination of need for MAP machinery. However, according to (Rooney, 2005), the optimum method of gas atmosphere modification of the product is in correlation with food/package combination and is not normally applicable across broad types of food products. Therefore, it can be concluded that no approach of O₂ scavenging/CO₂ emitting is likely to fulfill varying demands on beverage and food packaging.

Among the different types of microorganisms that are able to create MAP, probiotics will be further evaluated in this study. Absorbing oxygen, releasing CO₂, antioxidants and other reaction end products to the interior atmosphere of the package by probiotics not only create MAP, but also increase the nutritional value of the product (Altieria, et al., 2004); (Anthierens, et al., 2011); (Adams & Moss, 2008).

6.3 Bringing proposed product as a probiotic-containing functional food product to the market

Constantly growing consumer health consciousness and expenditure are socio-economic aspects responsible for increasing interest in functional food products (Sanders & Huis, 1999). Concerns about side-effects of pharmaceutical agents and consumers demand for natural product has stimulated development of probiotic containing functional foods (Reid, 2006). Probiotic containing products are already well accepted in the US and Japan and are gaining increasing acceptance and popularity all over the developed world. They are also relatively well established in Europe particularly in the dairy sector. While in Europe the key area of functional food market has been the development of probiotic dairy foods, in the US mineral and vitamin fortification of foods has been the key focus of development. Therefore, in contrast to the US, where probiotic foods are typically considered niche products, there is an advanced acceptance level of probiotics in Europe. There is a significant interest among the European consumers in food products claiming health benefits. However, health claim preferences by consumers vary in different European countries. (Stanton, et al., 2001)

In order to bring a probiotic containing functional food to the market different aspects including safety, functional and technological characteristics of the probiotic and labeling of the product have to be taken into consideration. Safety aspects include specifications of probiotic such as non-pathogenicity and origin characteristics of it. Functional aspects include strain selection, definition of probiotic activity and factors contributing to its biological activity; defining efficacious consumption level of it and its shelf life. Viability and persistence of probiotic strains in the GI-tract (Gastrointestinal tract) are crucial factors. They need to be able to maintain their functionality without creating off-flavors in the food product which they are incorporated to. (Saarela, et al., 2000)

In addition, factors related to the sensory aspects of probiotic foods are of the most important. Only by meeting consumer expectations the food industry can succeed to promote the consumption of functional probiotic products. However, before probiotic strains can benefit the consumer, they must primary be possible to manufacture at industrial scale. (Saarela, et al., 2000) Figure 6 represents the important considerations for probiotic strains supplemented into the food products. Considering all above aspects, selection procedure of probiotic strain for this project will be described in the next chapter.

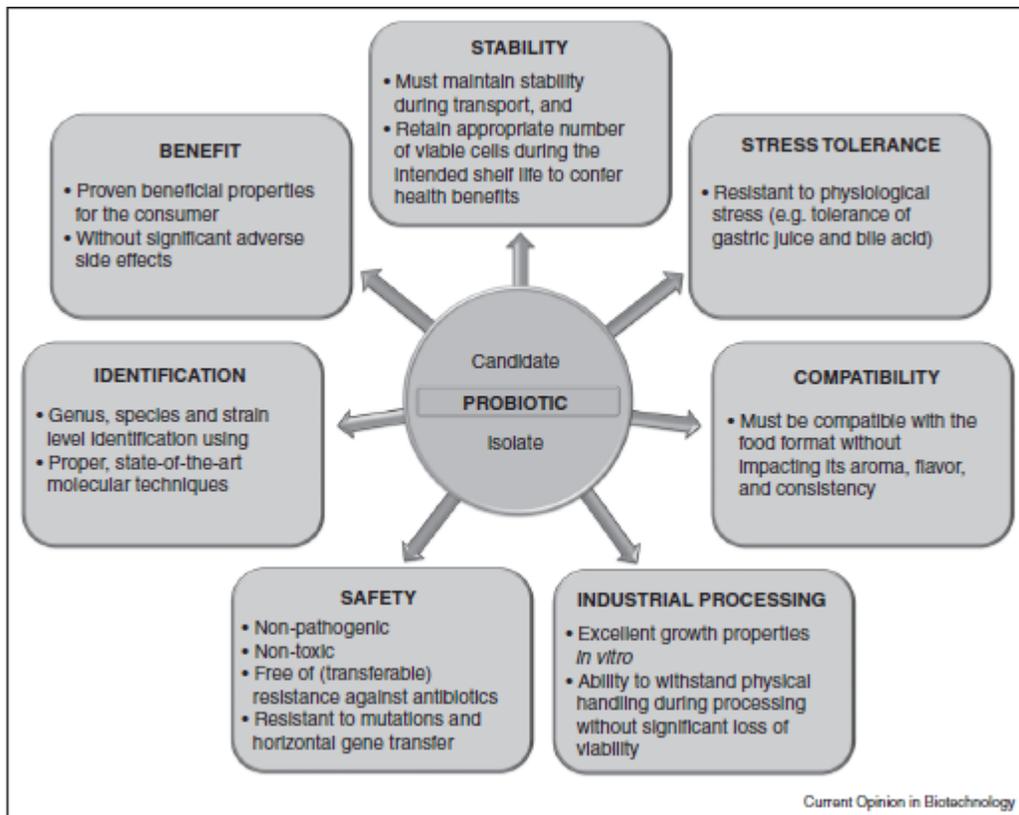


Figure 6: Important considerations for the selection of novel food probiotics (Rauch & Lynch, 2012)

6.3.1 Selection procedure of the potential probiotic to be placed inside the cap in this project through evaluating characteristics of different probiotics

Theoretical evaluation regarding safety and nutritional value of the product with the package containing *B. amyloliquefaciens* was done through literature review in the following paragraphs. However, further evaluation through experimental investigation is required. Although, according to Jonghe. D, et al (2009), the ability of *B. amyloliquefaciens* to ferment lactose was not determined, according to Welker & Campbell (1967) and Priest. F, et al (1987), it can ferment lactose, glucose, sucrose and fructose and produce weak acids. Optimum fermentation condition for this microorganism is temperature around 5°C and pH of 7 in controlled condition (Alam, et al., 1989). For optimal enzyme activity, depended on the favored enzyme type, special temperature range is required. As an example if we expect protease activity of *B. amylolyqufaciens* in a product, the optimal temperature may differ from another product which we prefer to increase its lipolytic enzyme activity. (Wizna, et al., 2008) Therefore, in the case of uncontrolled fermentation, quality and sensory properties of product after the process needs to be investigated by practical experiences.

It has been proved that fermentation by *B. amyloliquefaciens* results in increasing protein content, decreasing crude fiber and transforming limiting factors such as lignocelluloses to simpler molecule components in sago pith (Wizna, et al., 2008). *B. amyloliquefaciens* produces fibrinolytic enzyme against thrombo-embolism disease as a fermentation product in douchi which is a typical soybean fermented food in china (Peng, et al., 2003). This bacterium produces levan with efficient immunostimulant property giving 100% survival of infected carp with *Aeromonashydrophila* (Rairakhwada, et al., 2010). *B. amyloliquefaciens* has not been cited in any studies dealing with *Bacillus* sp. as infectious microorganisms and no invasive properties of this microorganism has been reported (Sietske de Boer & Diderichsen, 1991).

However, *B. amyloliquefaciens* was not selected as the optimal probiotic for creating MAP inside the cap because it is an aerobic microorganism. This microorganism does not grow under anaerobic condition which means that it needs to be in the form of inactivated spore before oxygen becomes available. This demands maintenance of the desiccated film of microorganism until its activation time (during period of distribution and storage) and also needs reactivation at the intended time. Therefore, in this project a facultative probiotic would be preferred to be incorporated inside the cap. In addition, despite identification of *Bacillus subtilis* species as QPS by European Food Safety Authority, spore-forming bacteria are not identified as GRAS by FDA to be used as probiotic. (Beek & Brul, 2010); (Thirabunyanon & Thongwittaya, 2011). However, it must be reminded that in the case of applying microorganisms into the package layer, *B. amyloliquefaciens* is the only spore forming probiotic microorganism evaluated in this report.

Lactobacillus reuteri was also one of the potential probiotics to be applied in the future experiments of this project and was evaluated in this report. This probiotic that resides in GI track of human belongs to the category of obligate heterofermentative species. It ferments prebiotics to

several end products including ethanol, lactate, acetic acid and carbon dioxide via phosphoketolase pathway. (Sinkiewicz, 2010) *L. reuteri*, which already has been used in the market such as in Biogaia's products, has several advantages including:

- Releasing reuterin which is an unique antimicrobial
- Well proved health benefits and safety
- Colonizing the entire GI tract
- Uniquely adapted to inhabit in the digestive tract of human (Rothschild, 1990)

More importantly it has been successfully used in commercial products for 22 years. However, despite the facultative anaerobic metabolism of this microorganism in the literatures, according to Molin. G, (professor of Food Microbiology at Lund University), it only tolerates aerobic conditions. In other words, this microorganism is not capable of efficient absorbing of oxygen in practice for the purpose of this project.

Therefore, the next potential probiotic *S. boulardii* matches our criteria to be the final choice of this project to be placed inside the cap. *S. boulardii* is facultative anaerobe, meaning that it can grow under both aerobic and anaerobic conditions (Czerucka, et al., 2007); (Line, et al., 2000). *S. boulardii*, which is classified as probiotic, is a subspecies of *saccharomyces cerevisiae* (Kühle & Jespersen, 2003); (Kühle, et al., 2005); (MacKenzie, et al., 2008). *S. cerevisiae* releases carbon dioxide in both aerobic and anaerobic environment (Oehlen, et al., 1994); (McKee & McKee, 2012); (Moat, et al., 2002). Therefore, *S. boulardii*, which has consistent microbiological and physiological characteristic to *S. cerevisiae*, is expected to produce CO₂ in both aerobic and anaerobic conditions (Kühle & Jespersen, 2003); (Piskur, J; professor of Molecular Genetics, Cell and Organism Biology at Lund University). In addition, CO₂ is among primary metabolites of facultative anaerobic yeasts in both aerobic and anaerobic conditions (Bekatorou, et al., 2006); (Querol & Fleet, 2006). Hence, it can be concluded that *S. boulardii*, which is facultative yeast, can release CO₂ inside the package for the purpose of this study.

S. boulardii is known for promoting the digestive health and may prevent acute childhood diarrhea, diarrhea associated with tube feeds and antibiotic-associated diarrhea. This non-pathogenic probiotic is one of the few yeasts that survives and does best at human body temperature of 37°C. (McFarland, 2010) *S. boulardii* has been used since the 1950s in Europe and except several sporadic cases of fungemia, which is likely to be related to catheter colonization, a remarkable safety profile by safety data during clinical tests has been collected for it. [Table 2](#) represents some examples of the commercially available *S. boulardii* containing products. (Czerucka, et al., 2007); (McFarland, 2010) Biocodex, which is a pharmaceutical company, supplies *S. boulardii* in two forms: freeze dried powder and an aqueous suspension, both forms in stationary phase (Graff, et al., 2008).

Application of a living organism in a product raises the potential risk in four general aspects: perseverance in the intestinal tract, translocation of the living organism from the intestine to other

body organs, transmission of antibiotic-resistance genes, and the progressing adverse reactions. In the case of *S. boulardii* the first three concerns have minimal risks. This probiotic does not develop any antifungal and antibiotic resistance. Also after oral ingestion was discontinued, it does not persist more than 3 to 5 days which eliminates the risk of persistence. (McFarland, 2010)

6.3.2 Activity of *S. boulardii* inside the package

Humidity is an important factor for growth of all microorganisms, including *S. boulardii*. The number of bacteria to achieve a substantial removal of oxygen in the package is related to the amount of present moisture and atmospheric volume within the container. (Hopkins & Banasiak, 1990). In order to evaluate possibilities of providing humidity for this yeast a review of previous studies has been done. In these studies humidity for the microorganisms is provided by different methods:

- I. In a study conducted by (Hopkins & Banasiak, 1990) *P. pastoris* was produced in the granulated, flaked or powdered form containing water content of 10 to 11 weight percent. Despite the moisture content of this powder, it was still dry to touch. In addition, to increase water content of this powder it was suggested that it be placed in an environment containing a source of moisture for 24 hours.
- II. In another study of applying *K. varians* or *P. subpelliculosa* into the packaging layer, humidity was provided by taking three steps. First, by adding water in the procedure of forming polymeric matrix, second, by pouring the solution of matrix on a polycarbonate plate and waiting for equilibrating at room humidity. Third, rehydration of the film a) when it is used as the interior layer of multilayer package of high humidity food or b) when it's used as a coating on a high humidity food. (Altieria, et al., 2004)
- III. In a patent by (Gist-Brocades, 1994) cells of *Saccharomyces cerevisiae* are covered by a food-grade layer of paraffin allowing only a very slow penetration of water. This yeast becomes wet and hence activated due to prolonged exposure to a water-saturated atmosphere in the head space of a water-containing product. This might be a procedure also applicable for *S. boulardii*.

Therefore, it can be concluded that the required humidity for activity of *S. boulardii* in the package can be provided by following different strategies.

6.3.2.1 Concerns regarding production and release of end-products by *S. boulardii* to the orange juice product

Produced gases such as carbon dioxide will pass through the covering gas permeable layer to be released to the head space of the package. Other reaction end products, which are not aimed to be released to the product until the point of consumption, may not cause volume expansion of the cap due to their trace amount. In addition, since the reaction end products are released to the product just prior to consumption, minimal changes in sensory properties of the product are

expected. However, changes in sensory properties of the orange juice, particularly when *S. boulardii* is added as the probiotic strain to the cap, need to be evaluated during the experimental tests.

Although carbon dioxide is desirable to be in interior atmosphere of some food products, it needs to be generated in varied concentrations to suit different specific foods. As an example, it should be optimized for each fruit type or fresh vegetable. (Kruijf, et al., 2010) Optimum concentration of CO₂ to create efficient MAP is dependent on the type of the product and the container's volume. To calculate this for orange juice, complicated prediction and deep knowledge of the microbiological metabolism of *S. boulardii* as well as environmental conditions of the product must be considered. However, the aim of this project has only been to make a qualitative theoretical investigation of CO₂ release to the product, not to conduct a quantitative assessment.

It is also worth mentioning that there are some probiotic containing packages used by Biogaia such as the drinkable yogurt called Orchard Maid with Probiotic Life Top Straw and products with lifeTop cap. LifeTop Straw releases the sensitive ingredients such as *L. reuteri* as the liquid passes through it at the time of consumption, whereas LifeTop™ Cap is a bottle closure containing sensitive components such as *L. reuteri* in a protective aluminum blister inside the cap. It releases powder of ingredients to the beverage when the blister breaks open at the time of pushing the top of the LifeTop™ Cap. In these products probiotics are only intended to be released to the product at the point of consumption to improve its nutritional quality regardless of any influence on gas atmosphere of the package. Therefore, in these products metabolic activity of the probiotics in the package is not important and they will start activity as they enter the intestinal cavity of the consumer. (Kerry & Butler, 2008)

In fact, the idea of this project and Biogaia LifeTop™ Cap both facilitate adding probiotics to wider variety of food products and allow probiotics to be remained unmixed with the beverage and be freshly mixed with the product as the consumer opens the can. However, in contrast to Biogaia's scope of only adding *L. reuteri* to the product, the aim of this project in future studies is to place *S. boulardii*, which in contrast to *L. reuteri* is capable of creating MAP, inside the cap. *S. boulardii* can be added to the package similarly to the Biogaia's products into the LifeTop cap or into other cap structures. These structures include Freshmix cap developed by Alto Company, Fusion cap by Portola Company, FreshCan Wedge developed by Ball Packaging Europe (BPE) and Degussa FreshTech Beverages LLC. However, the protective cover of this cap, which slits open at the time of consumption, should be replaced with a permeable layer to transform beverage to a functional drink upon packaging. The alternative covering layer must be permeable toward gases and allows a very slow penetration of water. [Figure 7]

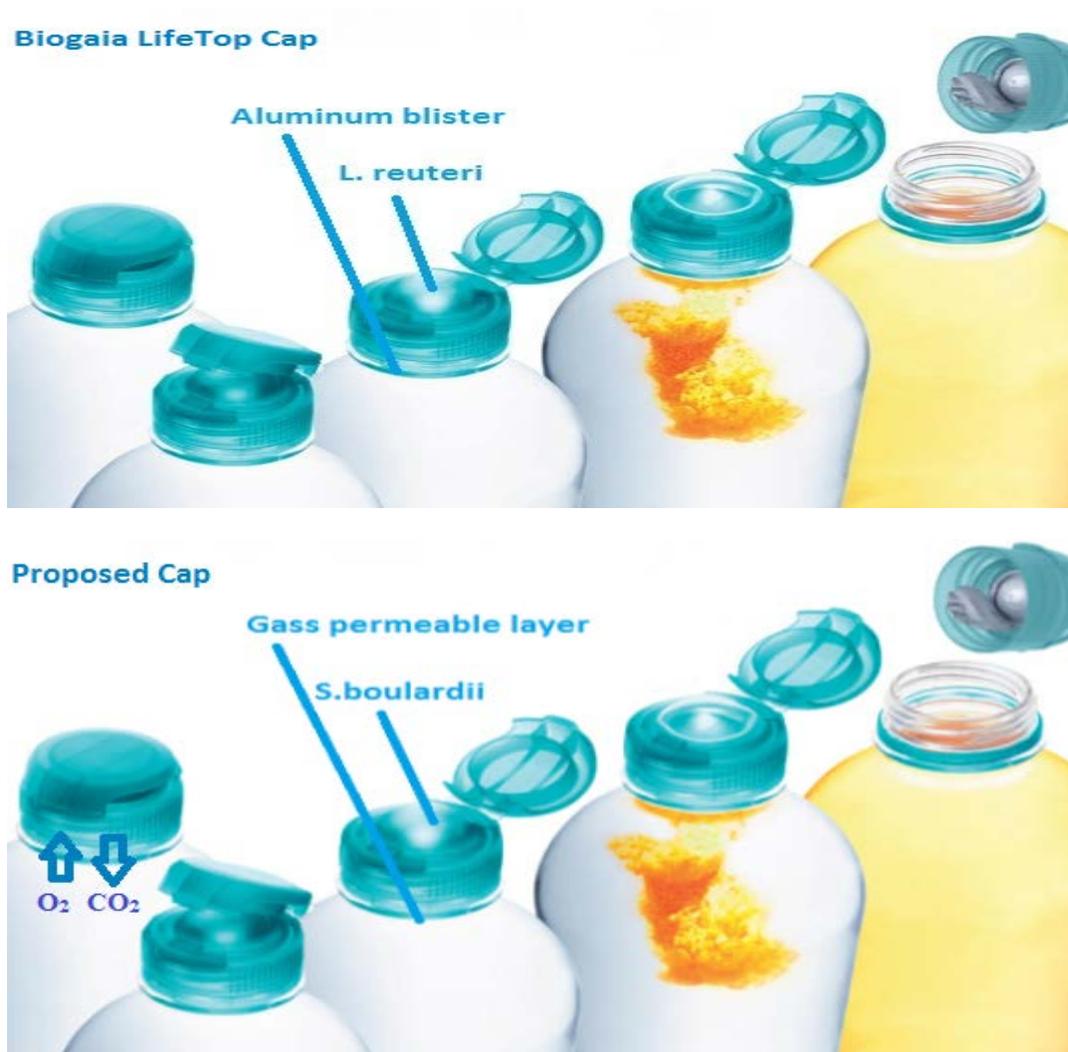


Figure 7: Biogaia LifeTop Cap vs. Proposed Cap

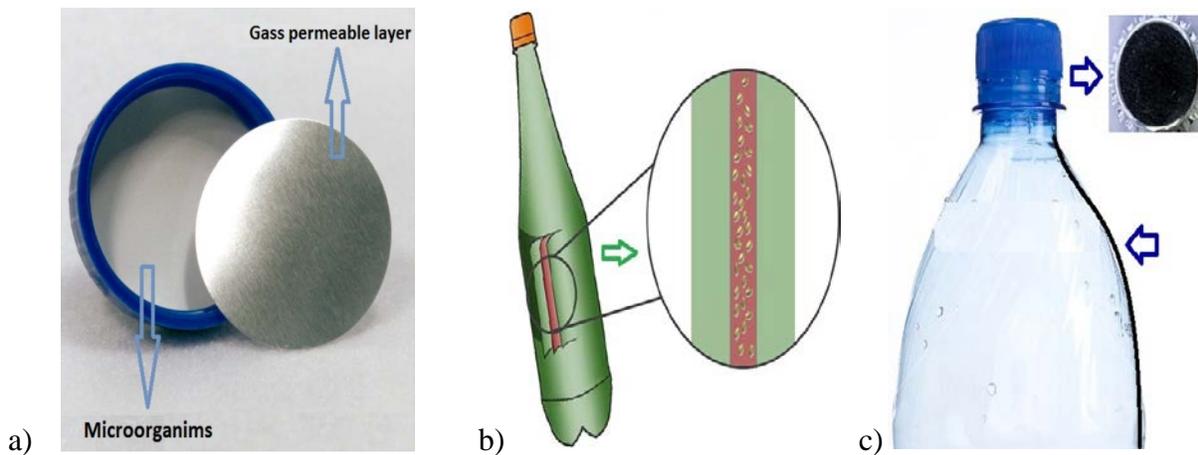
6.3.3 Microorganisms into a sachet, a cap, a closure liner or into the package layer?

As it has been discussed in previous chapters, microorganism can be applied to the package either as a sachet (6.1.1) or incorporated into the package structure (6.1.2). In both systems of incorporation, aseptic procedures should not affect the stability and performance of the microorganisms as active ingredient. To compare these two forms of incorporation, in contrast to sachet, active ingredients which are applied as a part of the package (into the cap, integrated into the closure liner or packaging layer), are prevented from an intimate contact with the food content. Therefore, active ingredients will not lose their functionality which is as a result of direct contact with high humidity product and aesthetic of the package will be enhanced. In addition, it might be more functional to target the problem at its source rather than let the oxygen permeate into the package and then scavenge it by an insert (Rooney, 2005). Therefore, it will be advantageous if microorganisms as active ingredient are applied into the package structure rather than into a sachet. However, probably due to low dosage of active ingredients incorporated into

the packaging layer or the closure liner, they are less effective compared to the sachet forms (Vermeiren, et al., 1999). Furthermore, there would be some limitations regarding integration of active ingredients, particularly live microorganism, into a package film (Anthierens, et al., 2011). Therefore, in order to achieve maximum efficiency, encapsulation of microorganisms, particularly *S. boulardii*, into the caps is of preference in this project. [Figure 8]



I. Incorporation of microorganisms into a sachet



II. Incorporation of microorganisms into the package structure; a) into the cap b) integrated as a separate layer into a multilayer package c) integrated into a uniform package layer and applied as either package wall or closure liner.

Figure 8: Microorganisms I. into the sachet, II. incorporated into the package layer

In addition, previous studies' entrapment of aerobic microorganisms as oxygen scavengers into the packaging layer have faced certain challenges. These limitations included complexity of forming an optimal polymeric matrix, survival of microorganisms in high temperature integration process and difficulties in providing nutritional requirements for the microorganisms in the package layer. Therefore, by placing MO into the cap it might be possible to overcome these

limitations in our project. Moreover, probiotic containing caps might be adaptable for a greater variety of packages than packaging films containing active components. [Table 3](#)

Table 3: Microorganisms into the closure vs. incorporated into the package film

	Packaging film	closure
Entrapment complexity	✓	×
Survival of MO	↓	↑
Activity of MO	↓	↑
Efficiency of the MO	↓	↑
Stability of the package	↓	↑

The closure will also contain either polyphenol or inulin as prebiotics. Prebiotics are complex carbohydrates which the human body is unable to digest but they provide nutritional requirements for the probiotics (Parkes, 2007). The closure can be fitted with a silicon valve to provide an airtight connection between cap and bottle. It prevents air from entering and eliminates the need for heat seal under the cap to improve survival of probiotics inside the cap. In addition, the covering layer of the cap is preferred to be food grade due to being in contact with both product and probiotics. Although limited contact between the orange juice and the probiotic containing cap is not harmful it is preferable that the container is kept upright during storage and distribution to minimize high permeation of humidity into the cap. In addition, it will be advantageous if probiotics do not exist on the sealing area between the covering layer and lip of the cap.

6.3.4 Consumer benefits, regulatory aspects and environmental impacts of the project

Consumer benefits: Modern consumers are progressively interested in their personal health. In Europe gut health is known to be the key sector for functional food products and the key growth segment has been the probiotic drinks. (Mattila-Sandholm, et al., 2002) The idea with this project regarding consumer’s benefit is extending shelf life, maintaining freshness and sensory quality of the orange juice whilst at the same time ensuring microbial safety and improving nutritional quality of it through probiotic containing packages. It may also provide convenience for people who use to take tablets of probiotics by providing a probiotic containing drink with extended

shelf life for them. However, eventually, it must be accepted by consumers and must be driven by real market demands rather than technological possibilities.

Regulations: In addition to the challenges of indicating the beneficial health effects of the probiotic containing products, manufacturers of probiotics need to prove that their products are in fulfillment with the regulations set by various government organizations to get approval for market release (Rauch & Lynch, 2012). Unfortunately, many of the available probiotic containing products in the market lack regulated quality control programs. Among 58 probiotic products from Europe, Asia, UK and Canada tested by Masco et al only 38% of them contained the dose claimed on the label. (McFarland, 2010)

There is little global unison among the regulatory authorities of probiotic containing products and they are regulated differently in different countries. As an example ESFA and FDA have very different standards of regulation for substantiating of health claim of a probiotic containing product by the manufacturers. (Rauch & Lynch, 2012) In the US, microorganisms which are approved as GRAS or as food additives are listed by the FDA and Federal Regulations. This list is also accepted as safe in Europe and Japan, however absence of a microorganism on this list doesn't imply it is unsafe to be used. (Sanders & Huis, 1999) On the other hand, In EU system of QPS (Qualified Presumption of Safety) is proposed by EFSA (European Food Safety Authority) for a pre-market safety assessment of applied microorganisms in the products. (Barlow, et al., 2007)

If the new type of package was developed in the US, different federal policies, which are regulated by specific centers within the FDA, apply to the product. In this regard, if there is no health benefit claim with the future probiotic containing package and it is only aimed to create MAP inside the package, the orange juice product fall under the control of the CFSAN (Center for Food Safety & Applied Nutrition, one of the centers within FDA). In the case of release of probiotic yeast and its end products in this project, *S. boulardii* needs be confirmed among GRAS list by FDA. Also if proposed probiotic containing cap claims a health benefit, Biologics Evaluation and Research (CBER) is responsible for overseeing the product. (Rauch & Lynch, 2012) .

In contrast, if the introduced cap is aimed to be developed in EU, different regulation of active packaging and probiotic containing product respectively by regulation 1935/2004/EC and EFSA must be meet. In this regard QPS status EFSA has been given to the baker yeasts including *S. boulardii* which means that it can be safely used in Europe with no need for further assessment. However, as well as *L. reuteri*, *S. boulardii* is not sufficiently characterized for its health claims. In fact, EFSA has been concluded that *S. boulardii* and *L. reuteri* are sufficiently characterized but a cause and effect relationship between their consumption and the claimed health benefits cannot be established (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012); (EFSA Panel on Dietetic Products, 2009); (Barlow, et al., 2007); (EFSA, 2009)

Environmental impacts: Packaging as an important part of the supply chain has an important effect on the environment and cost of the product. Glass has been used as a proper packaging material for many years. It provides better barrier properties than polymers particularly against gases but it is heavier and more expensive to be transported. The demand for shifting from glass and metal cans to plastic packs has introduced alternative packaging materials which provide good protection to the product at a reduced cost. (Rooney, 2005) Replacement of glass packaging with poly ethylene terephthalate (PET) resulted in diminishing the incidence of broken glass and reducing weight during distribution (Rooney, 2005). However, since PET cannot compete all of desirable properties of glass, (Rooney, 2005) proposed cap as active packaging can be used to upgrade its properties.

It is also worth to mention that compared to the vacuum packaging, more volume of the MAP adversely affects retail display, transportation and the environment (Kader, et al., 1989). Therefore, designing a package which is as efficient as MAP while having the shape of a vacuum package during the transportation might reduce the costs and environmental impacts. In addition, the potential of the proposed package as an oxygen scavenger for minimizing complexity in structure of multilayer plastics have favorable impacts on the environment, as long as does not adversely affect the manufacturing process. This is in line with conclusion by (Rooney, 2005) about oxygen scavengers.

Active MAP as an active packaging system may require an additional procedures resulting in more environmental impact. However, by providing longer shelf life of the product, the waste of food product and packaging material is expected to be reduced. Compared to the current packages in the market, although the proposed packages demand some additional procedures such as cultivation of microorganisms, it does not necessarily lead to additional environmental impact. Because the multifunctional proposed packages also appear as probiotic products in the market and all current probiotic products include cultivation of probiotics in their manufacturing processes.

Waste handling of the proposed packages: PET bottle consisting of the proposed packages can be recycled, incinerated or returned to the earth by landfill that environmental aspects need to be considered. A question that can be raised is that if PET bottles containing the proposed cap or the proposed packaging layer can be recycled by the consumer similar to the other types of PET bottles in the market. In this regard it needs to be mentioned that the content of the proposed cap, which is *S. boulardii*, is mainly consumed by the consumer. However, in the case of packaging film containing active ingredients of *B. amyloliquefaciens*, despite non-pathogenic and healthy character of this microorganism, environmental issues need to be considered by performing a life-cycle analysis before recycling.

The recycling process of PET bottles consists of four steps. These steps include: 1) processing post-consumer PET containers to the washed and dried flakes 2) drying and crystallization of PET flaks at high temperature 3) extrusion and re-crystallization of PET flaks at high temperature of 280 °C 4) solid state polymerization at high temperature of 200 to 240 °C. (EFSA Panel on

Food Contact Materials, 2012); (Awaja & Pavel, 2005) Therefore, although *B. amyloliquefaciens* endospores are able to survive process of incorporation into PETG at 210 °C (Anthierens, et al., 2011), applied temperature during the recycling procedure is above 210 °C.

In addition, *B. amyloliquefaciens* spores are expected to be germinated for activity inside the package prior to recycling. Hence, in their germinated state, they are much more susceptible to environmental stresses such as high temperature of recycling compared to the spores. Therefore, considering high temperature applied in the recycling procedure of PET bottles, no critical concern in recycling of the proposed packaging film containing spores of *B. amyloliquefaciens* is expected. Therefore, current recycling procedure of post-consumer PET bottle is concluded efficient enough to assure safe impact of the proposed packages on the environment.

In addition, incineration of the PET bottles, which typically takes place at temperatures around 700 °C, is effective enough to kill all microorganisms. Therefore, consumers are expected to be able to sort the post-consumer proposed packages for recycling similar to the current packages in the market. However, in order to guarantee the conventionality of the used materials, the Framework Regulation 1935/2004/EC requires the preparation of a compliance declaration which shall contain the following information: identity of the material; range of its application and the confirmation that the material acts in accordance with the requirements of the European guidelines. (Restuccia, et al., 2010)

6.3.5 Risk assessment and compliance of the proposed method

In the case of using functional barrier in a multilayer material, the following information shall be additionally provided for the authority: the identity of the substances of the functional barrier, the maximum heat treatment for the material and the date of latest use of it. An appropriate documentation to demonstrate the conformity of the material with the relevant requirements at each step of manufacture, processing, and distribution shall be also available. This certification may contain the results of the implemented analysis to demonstrate the conformity of the material and article particularly the compliance with the quantitative limitations such as OML, SML (overall migration limit, specific migration limit) in the use of the substances. The requirements related to components which are not listed in positive lists and the migration of the substances in detectable amounts set out in Framework Regulation 1935/2004/EC. The declaration of compliance should comprise three sections of summary, administrative part and technical dossier. The technical dossier should be including: an identity of the active substance and its physical or chemical characteristics, an overview of the intended application, the manufacturing process, an existing authorizations, toxicological information and migration data.

Migration data of active substances and, if any, their degradation and reaction products must be provided by means of proper conventional migration tests. If the total amount of substance, which is available to migrate, is less than migration limits, even if everything migrates to the food, the SML cannot be exceeded. On the contrary, if the value is above the migration limits,

mathematical modeling can be useful in order to measure how much of the components can potentially migrate to the food product. (Restuccia, et al., 2010)

6.3.6 Proposed idea and the distribution chain

Figure 9 represents contribution of Active packaging for retaining quality and freshness, reducing losses and adding unique properties to the product in different stages of the supply chain. The proposed method in this project as a part of active packaging can help the grower and the transporter to achieve optimum quality retention. It may also contribute simplification of the plant operations such as elimination of juice debittering operations to help the processors to differentiate their products which is in line with conclusions by Rooney (2005) about Active Packaging. This may result in cost saving on packaging operations but costs of process optimizations and active components need to be also considered. The potential benefits of the active packaging along distribution chain can be seen in figure 9.

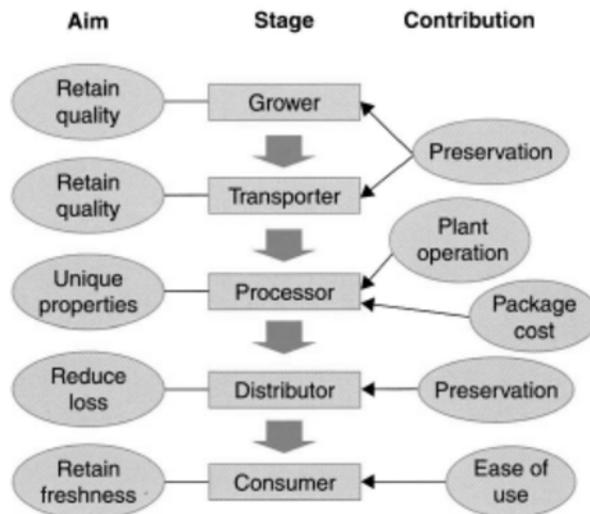


Figure 9: The distribution chain: targets and opportunities for active packaging. (Rooney, 2005)

The decision for selection of active packaging for a product is based on several factors. These drivers are including process engineering restrictions, economic benefits, convenience in use, environmental impacts and side effects resulting from some changes in the processing or packaging [Figure 10] (Rooney, 2005).

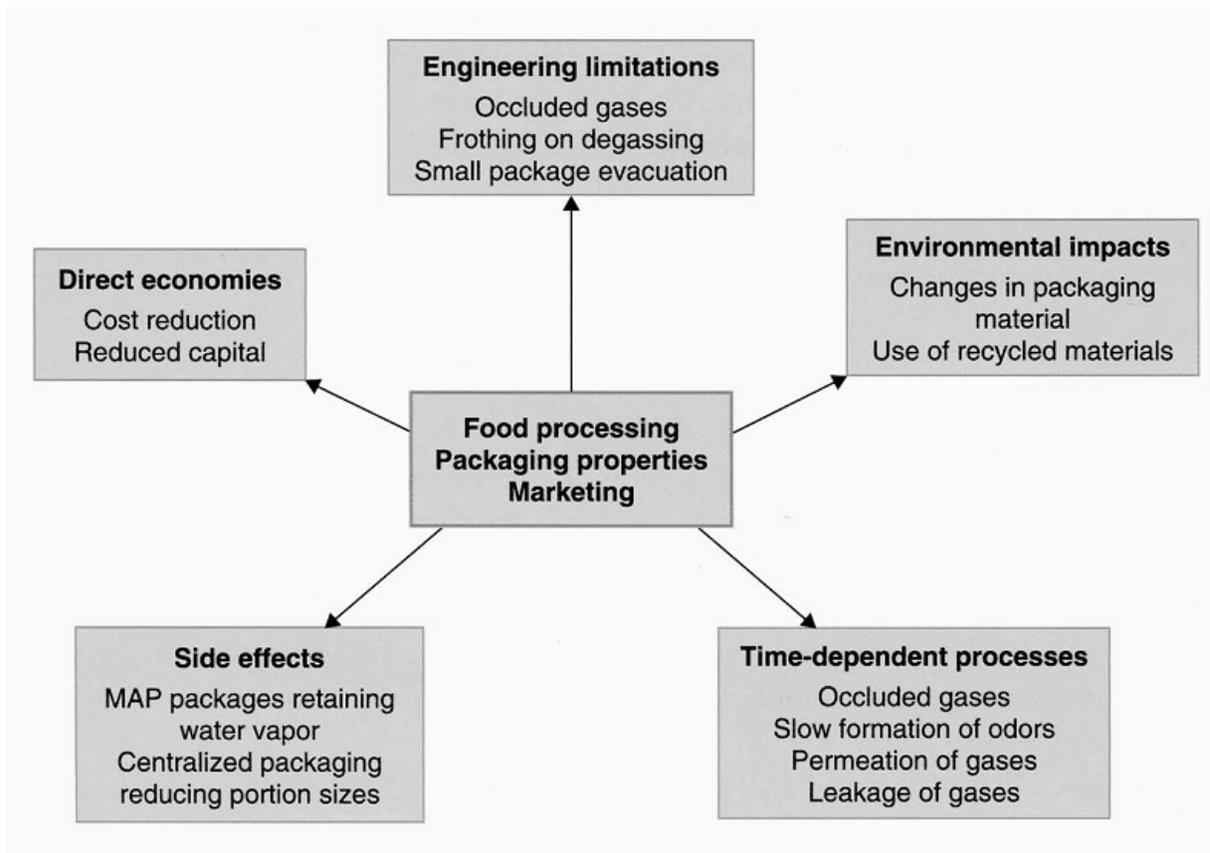


Figure 10: Current constrains on food processing, packaging and marketing (Rooney, 2005)

Economic advantages: In order to have an optimal package, the passive package may be coupled with packaging processes which introduces cost due to additional process requirements or line-speed limitation (Rooney, 2005). Introduction of *S. boulardii* as an active component for oxygen-sensitive products instead of evacuation followed by inert-gas flushing provide opportunity of removal of two steps. Hence, depending on the cost evaluation of the oxygen scavengers and their application processes it may result in economic benefits. However, active packaging may necessitate some level of adaptation at the stages of package fabrication or filling and sealing operations which costs (Rooney, 2005).

Process engineering restrictions: Existing processing equipment does not always fulfill process requirements for removal of dissolved oxygen in beverages due to frothing (Rooney, 2005). Hence removal of dissolved oxygen using proposed active cap can be an attractive feature. However, biological limitations in the manufacturing process of the proposed packages need to be considered. These criteria include entrapment complexities of microorganisms into the package structure, their survival during the integration procedure and formulating an efficient and active culture of them which need to be evaluated in the future studies.

Time-dependent processes: Release of gases occluded in the food products during storage and distribution cannot be controlled by conventional passive techniques such as vacuum packaging and MAP. Beside processes occurring in the product, time-dependent permeability of the

packaging film to the atmospheric gases, most importantly oxygen and vapor is a concern. It is influenced by relative humidity, temperature and air movement around the package (Rooney, 2005); (Kader, et al., 1989). While existing passive packaging is not responding to solve these problems, active packaging techniques are capable of improving the quality of PET bottles particularly for protection of beverages. (Rooney, 2005) However, activation of the microorganisms, which depends on the different factors such as their metabolism, might be time consuming. As an example it has been investigated that oxygen absorption by *B. amyloliquefaciens* starts only after 1-2 days while initial fermentation of *S. boulardii* takes about 1-3 days. (Anthierens, et al., 2011); (Heldesheim & Jacob, 2012)

Secondary effects: Centralized processing of the products such as diced fruits can change surface-to-volume ration of them. However, removal of fruits skin introduces more concern about microbial growth in the product. This brings challenges for the package engineers and provides opportunities for active packaging to protect the quality of the product. (Rooney, 2005)

6.4 Why beverages such as orange juice?

Over the past few years, consumption of processed food products especially juice has been increasing. Among citrus juices, orange juice is the most consumed one for its high content of vitamin C and its pleasant taste. (Ros-Chumillas, et al., 2007) Ascorbic acid (one form of vitamin C), which is one of the main quality factors of the orange juice, is affected by several factors including pH, salt, light, temperature and oxygen. High extent of ascorbic acid loss is mainly associated with the amount of oxygen dissolved in the juice and that initially present in the head space of the package. Ascorbic acid will be decomposed even in absence of oxygen. However, its decomposition rate under anaerobic conditions is lower than under aerobic condition. Oxygen also affects the quality attributes and ultimately shelf life of the fruit juice adversely by increasing browning and risk of bacterial growth. Browning reaction which is increased due to presence of oxygen follows ascorbic acid loss. The rapid removal of oxygen was investigated as a key factor in diminishing browning and sustaining a higher concentration of ascorbic acid over the long storage period of the packed juice. (Zerdin, et al., 2003) The main factors affecting oxygen level in the package are initial oxygen inside the package, permeability of the packaging film, leakage of the container and any microbial or chemical reactions within the product. (Pascall, et al., 2008)

Traditionally packaging tries to minimize exposure of beverages to oxygen by applying materials such as foil laminates in brick packs or glass which have high barrier properties. However, oxygen may permeate the package due to leakage during the storage or can be entrapped into the package at the time of sealing. (Dawson, 2003); (Galic, et al., 2011). Oxygen in the headspace of the package can also be removed by flashing an inert gas into the head space of the package (N₂, CO₂), or by vacuum sealing or both. Such systems are used in modified atmosphere packaging of food products including orange juice and other brewing industries. Approximately 90–95% of the oxygen present inside the package can be removed by this technology. This makes removal of the last traces of oxygen an expensive process. However, by use of oxygen absorbents the level of

oxygen can be actively reduced to less than 0.01%. (Pereira de Abreu, et al., 2012). Hence, oxygen scavenger can reduce the oxygen level to a much lower level than those attained by modified atmosphere packaging. (Zerdin, et al., 2003). Therefore, the most quickly deteriorative reactions in beverage such as oxidation, microbial growth and color alterations, which are as a result of oxygen presence (Dawson, 2003), can be controlled by activity of the proposed cap.

Furthermore, the beverage technology applies science of taste. Excluding five main tastes, all other tastes are as a result of sense of smell. Aroma notes in beverage which are associated with freshness of the beverage are generally burned off by pasteurization. (Kerry & Butler, 2008) Successful commercialization of newly developed non-thermally processed foods may necessitate extra functions of packaging and deep research on interactions between non-thermally processed foods and packaging materials (Galic, et al., 2011). Therefore, applying proposed cap which contribute prohibitory effect on growth of spoilage microorganisms may assist non-thermally processes to compete pasteurization of the beverage. Moreover, although the rate of browning and spoilage is also temperature dependent in citrus juice (Zerdin, et al., 2003); low temperature during transportation might not be a critical factor where oxygen scavengers contribute controlling browning and growth of spoilage microorganisms. Oxygen scavenging might be significantly more essential when the juices are distributed under ambient temperature than packs which are distributed chilled (Rooney, 2005) [Figure 11].

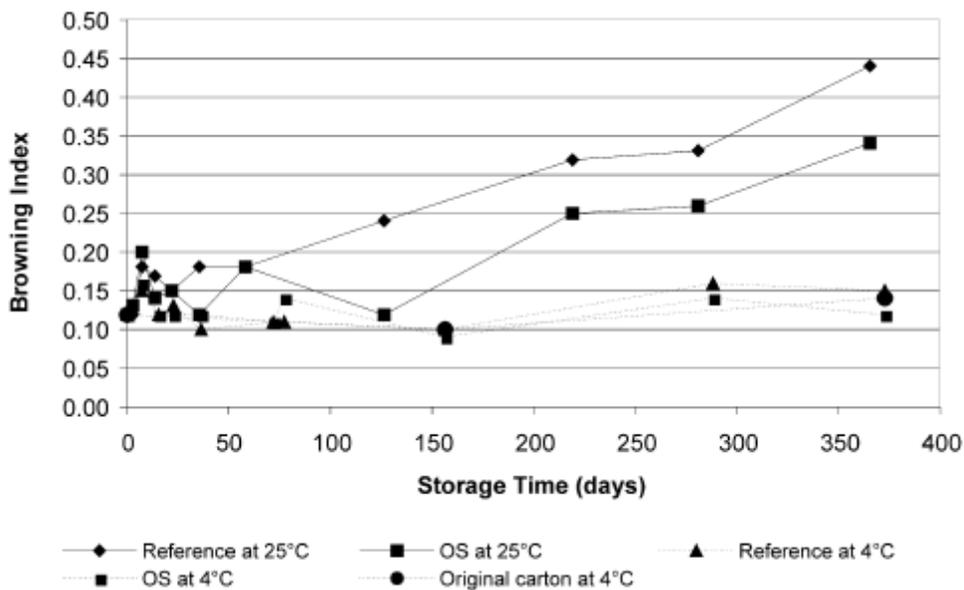


Figure 11: Browning of orange juice packed in OS and reference pouches stored at 25 and 4 °C. (Zerdin, et al., 2003)

Moreover, PET has good mechanical properties to be used in food packaging particularly liquid foods such as orange juice. However, in spite of low price of monolayer PET, it presents lower protection of ascorbic acid compared to multilayer PET and glass. (Ros-Chumillas, et al., 2007) Therefore, a package with improved barrier properties containing probiotic culture in the form of

sachet or as a package layer might be a good substitute for glass or expensive high-barrier films (Vermeiren, et al., 1999); (Krochta, 2003). In other words, orange juice packed with monolayer PET containing an oxygen scavenger combined with flashed nitrogen liquid in the head space and an aluminum foil seal in the screw-cap has been shown to provide much higher shelf life than those demanded on the market for juice aseptically packed in glass bottles. Therefore, the use of lower priced materials like monolayer PET in conjunction with techniques of reducing the presence of oxygen inside the package, appears as an attractive alternative for expensive and passive packaging materials for oxygen sensitive liquid product as orange juice. (Ros-Chumillas, et al., 2007) The new method not only by scavenging the residual and dissolved oxygen but also by providing an ongoing barrier to oxygen permeation provides extended protection of the juice from oxidative degradation. The achievable extension of shelf life is dependent on storage condition of the product, gas mixture of the head space and its ratio to the volume of the package. (Kerry & Butler, 2008) To develop a new and safe product it is necessary to understand the resulting effects on food and packaging and consequences of their interactions. Therefore, any decision should be proceed by specific laboratory tests. However, due to limitations of this project the idea was evaluated only theoretically.

7. Conclusion

Implementing of probiotic bacteria as acceptable ingredients by consumers, for creating modified atmosphere inside the package might be an interesting concept to the modern packaging technology. The present invention suggests a process which comprises the incorporation of *S. boulardii* capsulated or non-capsulated into the closure covered by a gas permeable layer constructed from fibers, papers, waxes and polymers such as paraffin. As a result the covering layer which is in contact with the head space of the orange juice allows these microorganisms to continuously modify the atmosphere of the package. The closure will also contain either polyphenol or inulin as prebiotics. The content of the cap will be drop to the single serve product at the time of opening the cap by the consumer. Therefore, the proposed cap is multifunctional by concurrent scavenging of oxygen, releasing of carbon dioxide and improving nutritional quality of the product. However, no approach of O₂ scavenging/ CO₂ emitting is applicable across broad types of food products. Therefore, the optimum method of gas atmosphere modification of the product will vary with the food/package combination.

The proposed cap can be applied in conjunction with current MAP technique or as a substitute for it. Although the latter case may allow higher packaging speed, production of probiotic containing closures might be time consuming and costly. Therefore, in order to design an optimal, cost-effective and safe package, much more information is needed on the action of these active ingredients in different environments. The proposed cap can help the manufacturer to create a distinct utility proposition from existing products to meet consumer expectations. However,

before probiotic strains can benefit the consumer, they must primary be possible to manufacture at industrial scale which demands more studies in the future.

8. Future studies

If it could be confirmed by future experimental studies that *S. boulardii* can successfully be used to create an oxygen poor-carbon dioxide rich atmosphere inside the package, (MAP), the project will further set out to investigate:

- Optimal volume of CO₂ to be released to the head space of orange package
- Possible interaction of the product and the *S. boulardii* containing cap to ensure
 - Absence of perceptible sensory alterations of the orange juice
 - Nutritional quality improvement of the product
 - Compliance with safety regulations of active packaging
- Optimal condition for activity of the *S. boulardii* inside the cap
- Effective dosage of *S. boulardii* to have a beneficial health effect on the product
- Influence of rehydration conditions, nutritional requirements and entrapping matrix on respiratory of the *S. boulardii* to find the optimal microorganism entrapment conditions
- Consumer acceptance, costs and logistic aspects of the proposed cap

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Table 1: List of some commercial oxygen scavengers. (Pereira de Abreu, et al., 2012)

Commercial product	Company
ActiTUF™	M&G Finanziaria s.r.l.
Aegis HFX Resin	Honeywell International Inc.
Aegis OXCE Resin	
Ageless®	Mitsubishi Gas Chemical
Amosorb®	ColorMatrix Group Inc.
Amosorb SolO2	
Celox™	Grace Darex Packaging Technologies
Desi Pak®	Süd-Chemie AG
Sorb-It®	
Tri-Sorb®	
Getter Pak®	
2-in-1 Pak®	
Cryovac® OS Film	Sealed Air Corporation
O2S®	Bericap GmbH und Co. KG
O-Buster®	Hsaio Sung Non-Oxygen Chemical Co., Ltd.
Oxbar®	Constar International Inc.
MonOxbar®	
DiamondClear®	
Bioka Oxygen Absorber Sachets	Bioka Ltd.
Bioka Oxygen Scavenging Film Laminate	
ATCO®	ATCO/Standa Industrie
Ciba® Shelfplus™ O2	Ciba Inc. (BASF)
Oxyguard™	Toyo Seikan Kaisha, Ltd.
ZERO2	CSIRO
valOR Activ100	Valspar Corporation
valOR ActivBloc100	
Wonderkeep	Powdertech, Co. Ltd.
Powdertech's	
FreshMax®	Multisorb Technologies
FreshPax®	
FreshPax® CR	
FreshCard®	
StripPax®	
MiniPax®	
Freshilizer®	Toppan Printing Co.
Tri Sorb EVA	Tri-Seal (Tekni-Plex)
Tri Shield Tri Sorb EVA	
Tri Shield EVA blue	

Table 2: Examples of commercially available probiotics containing *Saccharomyces. boulardii* (McFarland, 2010)

Product name	Probiotic strain	Manufacturer	Stable at room temp ¹	Facility certified	Strain confirmed	Colony forming unit (cfu) per mg capsule or mL	Original strain-specific studies	Degree of clinical evidence ²
Single strain								
Florastor [®] (US) Perenterol [®] (Germany) Reflor [®] (Turkey) Ultra-Levure [®] (Asia) ³ SB+MOS	<i>S. boulardii</i> lyo	Biocodex (France)	Yes	EU GMP	Microsatellite sequencing polymorphism ^[28,29]	5 × 10 ⁹ /250 mg	Multiple ^[37,42]	A
	<i>S. boulardii</i>	Jarrow Formulas (Los Angeles, CA) and Gnosis (Italy)	No	EU GMP	Not stated	1.5 × 10 ⁹	One study ^[63]	C
<i>Saccharomyces boulardii</i>	<i>S. boulardii</i>	Kirkman (Oregon)	No	GMP	DNA fingerprint	3 × 10 ⁹ /150 mg	None	F
<i>Saccharomyces boulardii</i>	<i>S. boulardii</i>	Allergy Research Group (CA)/NutriCology (CA)	No	GMP	DNA fingerprint	3 × 10 ⁹ /150 mg	None	F
<i>Saccharomyces boulardii</i>	<i>S. boulardii</i>	Klaire Labs (Reno Nevada)	No	GMP ISO 9001	Ribotyping	3 × 10 ⁹ /150 mg	None	F
In mixtures of probiotics								
Protecfior [®] (Canada) Erce Flora [®] (Belgium)	<i>S. cerevisiae boulardii</i> CNCM I-1077) + <i>L. rhamnosus</i> + <i>L. acidophilus</i> + Bifido. strain	Institut Rosell-Lallemand (Quebec, Canada)	Not stated	Canadian GMP	Not stated	10 ⁹ /2 mL	Two studies ^[64,65]	D
MitoMix [®]	<i>S. boulardii</i> and <i>Pediococcus acidilactici</i>	Imagilm Technology (Maryland)	Yes	Not stated	Not stated	2.3 × 10 ⁹ /capsule	One study ^[66]	D
Primal Defense™	<i>S. boulardii</i> with 13 other strains ⁴	Garden of Life (FL)	Yes	Not stated	No (only certified "organic")	Total 1 × 10 ⁹ per 410 mg	None	F
Pro-Bio Defense™	<i>S. boulardii</i> + 7 other strains ⁵	Kirkman (OR)	No	GMP	DNA fingerprint	Total: 2 × 10 ¹⁰ /cap, no data on SB cfu	None	F
ABX Support™	<i>S. boulardii</i> + <i>L. rhamnosus</i> + <i>Bifido. bifidum</i> + <i>Bifido. breve</i>	Klaire Labs (NEV)	No	GMP ISO 9001	Ribotyping	5 × 10 ⁹ /300 mg	None	F
Kombucha fermented tea	<i>S. boulardii</i> + <i>L. bacterium</i> + blue-green algae	Millennium Products, Inc.	No	No	Not stated	1 × 10 ⁹ per 16 oz.	None	F

¹Products not stable at room temperature require refrigeration, probably heat-dried. Products stable at room temperature are typically packaged in blister packs and are lyophilized; ²Degree of evidence: A = at least two randomized, controlled clinical trials in patients; B = one randomized controlled clinical trial in patients, C = case reports, uncontrolled randomized trials or open safety/kinetic studies in patients or volunteers, D = *in vivo* animal model or *in vitro* studies only, E = expert opinion only, F = no direct evidence; ³A few examples, Biocodex has over 40 brand names worldwide; ⁴Other strains in Primal Defense include: 11 strains of *Lactobacilli* (*L. acidophilus*, *L. bulgaricus*, *L. lactis*, *L. plantarum*, *L. casei*, *L. lactis*, *L. leichmannii*, *L. brevis*, *L. caucasicus*, *L. fermenti*, *L. helveticus* and 1 strain each: *Bifidobacterium bifidum*, *Bacillus subtilis*, *Bacillus licheniformis*); ⁵Other strains in Pro-Bio Defense include: 5 strains of *Lactobacilli* (*L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*) and *Bifidobacterium lactis* and *Streptococcus thermophilus*. GMP: Good manufacturing practices.